Nitric Oxide As A Regulator Of Apoptosis And Inflammation In Human Skin Following Ultraviolet Irradiation

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Declaration

I hereby declare that the data presented in this thesis is the results of my own work, carried out under the supervision of Dr R Weller at the Department of Dermatology and Dr AG Rossi at the Department of Inflammation Research, the University of Edinburgh.

I declare that this thesis was composed entirely by myself.

I declare that this thesis has not been submitted for any other degree, postgraduate diploma or professional qualification.

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Dedication

I dedicate this thesis to Phil, my husband and best friend.

Abstract

The importance of inorganic nitrogen oxides has been known since 800AD. However, it was not until the 1980s that major scientific advances were made in this area of clinical science. Since the finding that NO released from smooth muscle vascular endothelium has vasodilatory properties, there has been a surge in research designed to further elucidate the biological roles of this free radical.

Enzyme-dependent and enzyme-independent mechanisms of NO generation have been observed in human skin. NO generation by both mechanisms increases following ultraviolet radiation (UVR). *In vivo* murine data suggests that NO has an anti-apoptotic role in human skin following UVR.

In the work presented in this thesis I have shown that zeolite NO (Ze-NO) is an inert topical NO donor which releases physiologically relevant concentrations of NO. The NO released by Ze-NO induces minimal cutaneous inflammation, in contrast to earlier NO donors investigated. I provide evidence to show that NO is stored in the form of NO-related products in human skin and sweat on the skin surface. NO is released from the products within 30 minutes of exposure to UVR. I provide preliminary *in vivo* human data which suggests that NO is acting anti-apoptotically in human skin following UVR. NO had no significant effect on DNA damage or repair following UVR. Finally, I provide evidence that a control mechanism for the upregulation of iNOS exists in human skin in the form of arginase and the urea cycle.

In order to develop our understanding of the role of NO in human skin, all the work presented in this thesis has been carried out *in vivo* in human subjects. NO has complex pleiotropic actions which depend upon the concentration of NO, the target cell and the microenvironment. *In vivo* clinical research, using physiologically relevant parameters, is therefore essential if we are to further our knowledge of this free radical.

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A huge thanks must go to all study volunteers. All the studies within this thesis have focussed on *in vivo* clinical research. None of the studies would have been possible without the willingness, enthusiasm and dedication of all the volunteers involved.

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Publications

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Presentations (no abstract)

Nitric oxide is not as potent an inflammatory mediator in human skin as previously indicated. Mowbray M, Tan X, Weller R. 2006 Scottish Skin Biology Club, Edinburgh (Oral Presentation).

Quantification of enzyme independent stores of nitric oxide in human skin. Mowbray M, Lomatschinsky N, Jones S, Weller R. 2006 SDS Annual Meeting, Glasgow (Oral Presentation) *Awarded 'best oral presentation'.

Nitric oxide is not as potent an inflammatory mediator in human skin as previously indicated. Tan X, Mowbray M, Morris R, Weller R. 2006 SDS Annual Meeting, Glasgow (Oral Presentation).

UVB upregulates arginase in the epidermis via NO release. M Mowbray, L Van Der Loop, N Bredenkamp, L Declerq, R Weller. 2007 SDS General Meeting, Edinburgh (Oral Presentation).

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UVA induces rapid photolysis of NO stores in human skin. M Mowbray, R Weller. 2007 SDS AGM, Dunfermline (Oral Presentation).

Contents

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List of Abbreviations

6-4PP	6-4 photoproduct
BB UVB	broad band UVB
Ca	Calcium
CAT	cationic amino acid transporters
cGMP	cyclic guanosine monophosphate
cLE	cutaneous lupus erythematosus
cNOS	constitutive nitric oxide synthase
Co	Cobalt
CPD	cyclobutane pyrimidine dimer
DAB	3,3'diaminobenzidine
DEA	Diethylamine
DETA	Diethylenetriamine
DNA-PK	DNA-dependent protein kinase
EDRF	endothelial derived relaxing factor
EDTA	Ethylenediamine tetra acetic acid
eNOS	endothelial nitric oxide synthase
FAD	flavine adenine dinucleotide
FMN	flavine mononucleotide
H&E	haematoxylin and eosin
Hb	Haemoglobin
Hg	Mercury
HgCl2	mercuric chloride
HPLC	high-pressure liquid chromatography
I2	Iodine
IFNγ	interferon γ
IHC	Immunohistochemical
IHD	ischaemic heart disease
IL-1β	interleukin 1β
IL-8	interleukin 8
iNOS	inducible nitric oxide synthase
ISMN	isosorbide mononitrate
ISNT	in situ nick-translation
KI	potassium iodide
LE	lupus erythematosus
LPS	Lipopolysaccharide
LTA	zeolite-A
MED	minimal erythemal dose
Mn	Manganese
MRL/lpr mice	murine model of lupus
N2O3	dinitrogen trioxide, nitrous anhydride
NaCl	normal saline
NAd	Noradrenaline
NADPH	nicotinamide adenine dinucleotide
NB UVB	narrow band UVB
NEM	N-ethylmaleimide
NER	nucleotide excision repair
NMSC	nonmelanoma skin cancer
nNOS	neuronal nitric oxide synthase
NO	nitric oxide

NO-	Nitroxyl
NO+	Nitrosonium
NO2	nitrogen dioxide
NO2-	Nitrite
NO3-	Nitrate
NOS	nitric oxide synthase
Nox	nitric oxide oxidation
O2-	Superoxide
O3	Ozone
OA	Osteoarthritis
ONOO-	Peroxynitrite
PARP	poly (ADP) ribose polymerase
PP	Photoproduct
RA	rheumatoid arthritis
RNNO	Nitrosamine
RNS	reactive nitrogen species
ROS	reactive oxygen species
RSNO	Nitrosothiol
RT	room temperature
SED	standard erythemal dose
SH	suphydryl group
SLE	systemic lupus erythematosus
sNOAlb	S-nitrosoalbumin
SNO-Hb	S-nitrosated haemoglobin
SNP	sodium nitroprusside
SPER	Spermine
SSc	systemic sclerosis
Th2	T helper 2 cells
TLR4	toll-like receptor 4
TNFα	tumour necrosis factor α
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling
UVA	ultraviolet A
UVB	ultraviolet B
UVC	ultraviolet C
UVR	ultraviolet radiation
XP	xeroderma pigmentosum
Ze	Zeolite
Ze-NO	zeolite NO donor

CHAPTER 1

GENERAL INTRODUCTION

1. CHAPTER 1 - GENERAL INTRODUCTION

1.1. The history of nitric oxide

Knowledge of the beneficial and/or physiological effects of inorganic nitrogen oxides has a long and rich history. The most ancient example is the use of nitrate (NO_3^-) salts to cure foods, this colours meats and also acts as an antibacterial against botulism(Binkerd and Kolari, 1975). Documents from around 800AD suggest that NO_3^- and nitrite (NO_2^-) were used in Chinese traditional medicine to relieve 'acute heart pains, and cold in the hands and feet'(Gladwin et al., 2005). In 1865 Brunton discovered the vasodilator 'amyl nitrite' as a treatment for angina(Lauder Brunton, 1867). From the time of discovery of the vasodilatory properties of the organic nitrates and nitrites, it took more than 100-years to elucidate their mode of action at the molecular level. The vasodilator action of these 'nitrovasodilators' is now known to be mediated by the release of nitric oxide (NO).

The growth of interest in the biological effects of NO began in 1980. Furchgott and Zawadzki found that strips of aorta with intact endothelium relaxed in response to acetylcholine, but constricted in response to the same agonist when the endothelium had been rubbed off, indicating an essential role for the endothelium in smooth muscle vascular reactivity(Furchgott and Zawadzki, 1980b). The molecule mediating this effect was termed endothelium-derived-relaxing-factor (EDRF). In 1987 Furchgott and Ignarro independently proposed that EDRF is NO(Ignarro et al., 1987a;Furchgott, 1986), subsequently Ignarro and Palmer showed that EDRF and NO are indistinguishable based on key chemical properties(Ignarro et al., 1987c;Palmer et al., 1987). Studies have shown that NO released from the endothelium diffuses into vascular smooth muscle, where it activates soluble guanylyl cyclase by binding to its haem group, which results in increased cyclic guanosine monophosphate (cGMP) levels(Ignarro et al., 1986). Cyclic GMP activates GMP-dependent kinases and downstream signalling that ultimately decreases intracellular calcium concentration leading to vasodilation(Ignarro, 1990).

In 1998 Robert Furchgott, Louis Ignarro and Ferid Murad won the Noble Prize in Physiology or Medicine, for their work on the role played by NO in regulating blood pressure and blood flow. The prizes are presented on the 10th of December, the anniversary of the death of Alfred Nobel, the 19th century Swedish industrialist and inventor. Ironically, Alfred Noble is best known for the discovery of dynamite, which he formed by incorporating the now well known NO-donor drug glyceryl trinitrate into kieselguhr, a porous material consisting largely of silica.

1.2. Free radical properties of NO

Oxygen and nitrogen together comprise over 98% of the air we breathe. Both play a central role in biology. NO is the simplest compound of nitrogen and oxygen. NO is a diatomic free radical molecule and is a gas at room and body temperature. As a free radical NO is a highly reactive molecule within biological systems, reacting with other free radicals, molecular oxygen and transition metals. NO is soluble both in water and lipid(Henry et al., 1993), it is therefore, freely diffusible in the environment of the cell. The reactivity of NO is related to its redox properties, as is oxygen, both forming redox couples. Oxygen-derived species are termed reactive oxygen species (ROS), whereas those derived from nitrogen are termed reactive nitrogen species (RNS). Some RNS may be useful but can be toxic in excess, for example NO. Other RNS are probably always damaging, for example nitrogen dioxide (NO_2) and peroxynitrite ($ONOO^-$). The same is true for ROS; superoxide is often useful (O_2^-), but the hydroxyl radical (OH) is probably always detrimental. It is the balance between NO and ROS that determines their biological action.

In mammalian blood and tissues NO may react with molecular oxygen to form NO_2^- or with O_2^- to form $ONOO^-$, which subsequently isomerises to yield NO_3^-. Alternatively, NO may react, probably via dinitrogen trioxide (N_2O_3), with thiols and amines to form nitrosothiols (RSNOs) and nitrosamines (RNNOs) (figure 1.1).

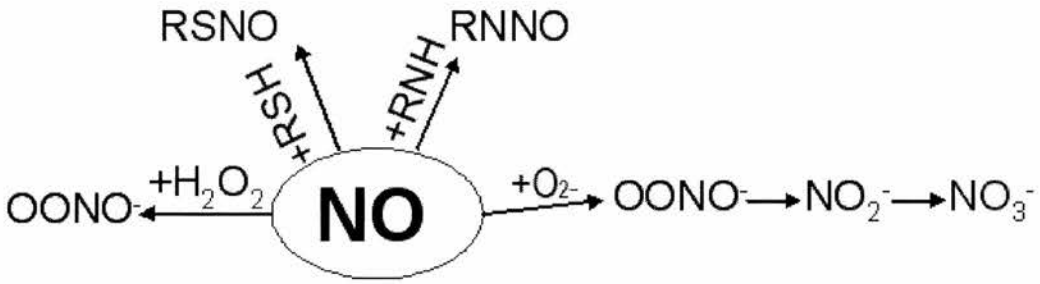


Figure 1.1 *Potential reactions of NO in blood and tissues*

Despite its structural simplicity, NO has a complex chemistry, endowing the free radical with wide and varied biological actions. NO is synthesised by a number of cell types, where it acts as a highly regulated autocrine and paracrine signalling molecule. The sphere of influence of NO itself is likely to only extend to several cell diameters (~100μm) of its origin, on account of its reactive nature. Thus, NO is often described as a local mediator that does not need complex metabolism for clearance; it is simply diluted and then oxidised to nitrite and nitrate over time(Miller and Megson, 2007c).

1.3. Synthesis and transport of NO

1.3.1. Enzyme dependent NO synthesis

NO is generated by the enzyme nitric oxide synthase (NOS)(Moncada and Higgs, 1993), which catalyzes the conversion of L-arginine to L-citrulline and NO with a 1:1 stoiochiometry(Griffith and Stuehr, 1995). Three different NOS isoforms exist that are encoded by different genes located on different chromosomes. Two enzyme isoforms are constitutively expressed (endothelial and neuronal cNOS), whereas one isoform is an inducible enzyme (iNOS), initially found in macrophages(Knowles and Moncada, 1994). All NOS isoforms show moderate homology with cytochrome p450 reductase and exist in their active form of homodimers of two subunits with molecular masses of approximately 135 kDa (eNOS), 150-160 kDa (NNOS) and 130 kDa (iNOS)(Bruch-Gerharz et al., 1998). All isoenzymes require the cofactors reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN), flavin dinucleotide (FAD), protoporphyrin IX haem,

tetrahydrobiopterin and calmodulin(Cho et al., 1992;Kroncke et al., 1995a;Nathan and Xie, 1994). Differences exist in the cellular distribution, regulation and enzymatic activities of the three NOS isoforms.

The cNOS isoforms, which were first detected in neuronal cells (nNOS) and endothelial cells (eNOS), are active only after binding of calcium-calmodulin complexes, and produce picomolar to nanomolar amounts of NO for short periods of time (seconds to minutes). NO synthesised by these isoforms usually acts as an intercellular signalling molecule mediating time-restricted events. cNOS isoforms are generally regarded as providing regulatory and homeostatic functions such as limiting ultraviolet-induced epidermal apoptosis, maintaining blood pressure, inhibiting platelet aggregation and leukocyte adhesion, and promoting gastrointestinal motility(Abramson et al., 2001).

In contrast to the cNOS isoforms, inducible NOS (iNOS) is regarded as playing a more pathological role. iNOS is an inducible and Ca independent isoenzyme. iNOS is transcriptionally regulated and therefore requires *de novo* messenger RNA and protein synthesis for its activity(Kroncke et al., 1995b). It is expressed following stimulation with agents such as proinflammatory cytokines, bacterial endotoxins and ultraviolet radiation, and generates significantly greater amounts of NO for comparatively long periods of time (hours-days)(Kolb and Kolb-Bachofen, 1998a).

All three NOS isoforms have been isolated in human skin(Baudouin and Tachon, 1996;Becherel et al., 1994;Bull et al., 1996;Sakai et al., 1996;Wang et al., 1996;Romero-Graillet et al., 1996). Expression of nNOS has been found in keratinocytes and melanocytes, whereas expression of eNOS appears to be restricted to endothelial cells(Baudouin and Tachon, 1996;Dippel et al., 1994;Goldsmith et al., 1996;Romero-Graillet et al., 1996;Weller, 1997). iNOS has been found to occur in most cells of the skin, including keratinocytes, Langerhans cells, fibroblasts and endothelial cells (figure 1.2)(Arany et al., 1996;Becherel et al., 1994;Bruch-Gerharz et al., 1996;Bull et al., 1996;Nameda et al., 1996;Qureshi et al., 1996;Sirsjo et al., 1996;Wang et al., 1996).

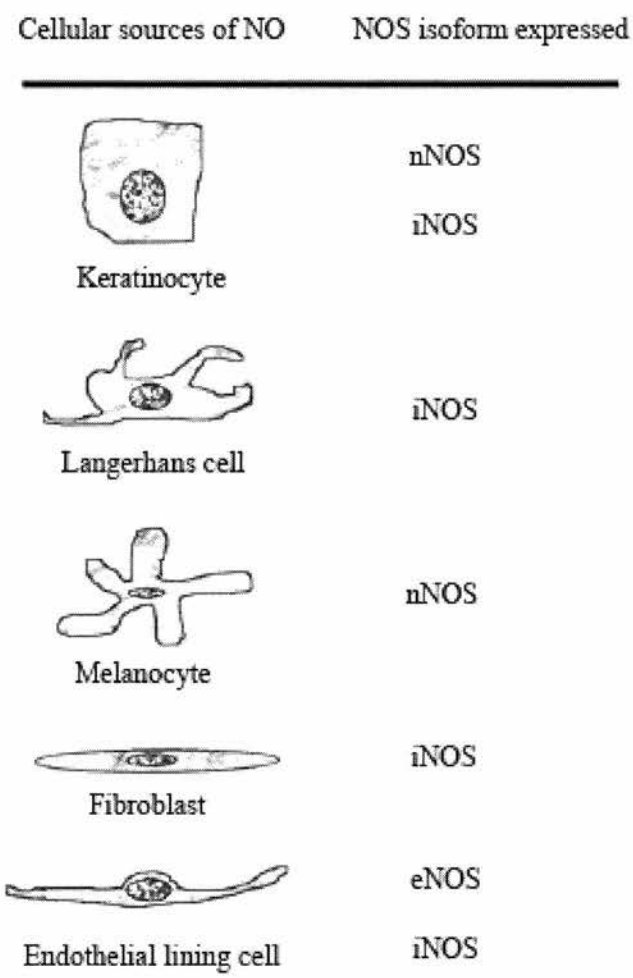


Figure 1.2 ***NOS expression in human skin***

1.3.2. NOS, arginase and the urea cycle

NOS catalyses the hydroxylation of the nitrogen in the guanidine group of L-arginine producing NO. This incorporates molecular oxygen into NO and L-citrulline. The byproduct of the reaction, L-citrulline, is recycled back to L-arginine as part of a modified urea cycle (figure 1.3)(Hecker et al., 1990). This has two functions:

- A secretory role to regenerate L-arginine for NO synthesis.
- An excretory role to eliminate excess nitrogen produced by the cell's metabolism.

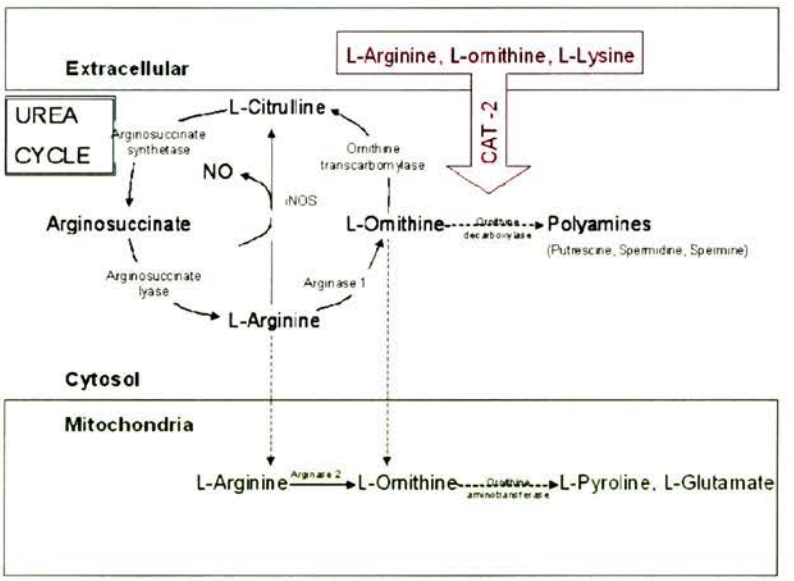


Figure 1.3 *The Urea cycle*

indicates potential for up regulation by UVR.

NO production following the up-regulation of iNOS is dependent on the availability of L-arginine, in addition to expression and activity of the cationic amino acid transporters (CAT), which allow L-arginine transport into cells. Normally, levels of L-arginine are sufficient for continuous secretory NO biosynthesis. However, it has been shown that in cytokine-activated endothelial cells, L-arginine supply at physiological levels can restrict eNOS activity(Suschek et al., 2003a). A tight link exists between iNOS and the urea cycle. Arginase 1 also competes for L-arginine as a substrate which it hydrolyses to ornithine and urea as part of the urea cycle (figure

1.3). Substrate competition by arginase 1 has been shown to be effective in down regulating NO production(Mori and Gotoh, 2000).

1.3.3. Enzyme independent NO synthesis

The t1/2 of NO (0.05-1.18milliseconds)(Borland, 1991;Liu et al., 1998;Vaughn et al., 2000) is very short, and thus it was initially regarded as having actions only in close proximity to its site of production. It is now accepted that NO can form additional bioactive stable carriers/donors in the blood and tissues by reacting with sufhydryl (SH) groups to form S-nitrosothiols (RSNOs)(Stamler et al., 1992b). These regulate proteins by S-nitrosation of specific cysteine residues(Hess et al., 2001). A major proportion of endogenous NO is also inactivated by oxidation to NO_2^- and the more stable NO_3^-, this inactivation may be transient as NO_2^- can itself be recycled into bioactive NO(Lundberg and Govoni, 2004). These more stable NO carriers/donors can be considered as biological NO stores, under the heading of NO-related products they include: NO_3^-, NO_2^-, RSNOs and N-nitrosoamines (RNNOs).

Nitrite has been shown to act as a signalling molecule in its own right(Bryan et al., 2005), and can also provide a bioavailable pool of NO during periods of hypoxia and ischaemia(Wink et al., 1996a;Zweier et al., 1995). Low molecular weight RSNOs exert NO-like activity *in vivo*(Ignarro et al., 1981) and circulating RSNOs can release NO when required(Rassaf et al., 2004).

In human skin the presence of NO-related products has been demonstrated both in sweat on the skin surface(Weller et al., 1996) and in the skin itself(Paunel et al., 2005). Using *ex vivo* skin biopsy specimens Paunel *et al.,* have shown that ultraviolet A (UVA) irradiation induces photolysis of RSNO and NO_2^- stored in human skin with subsequent high-output NO formation. This reaches a maximum 20 minutes after the onset of irradiation(Paunel et al., 2005). Weller *et al.,* have also described NO generation by sequential reduction of sweat NO_3^- to NO_2^- and then NO on the skin surface(Weller et al., 1996). While initially described on the skin surface, and in

the stomach, this NO_3^- reductive process may be common to many epithelial and mucosal surfaces.

1.3.4. NO transport

There are three proposed mechanisms for NO transportation in the bloodstream:

- *Compartmentalisation theory* - NO remains intact in the blood vessel for longer than initially believed. NO that diffuses into the vessel lumen and into the erythrocyte reacts at a nearly diffusion-limited rate with oxyhaemoglobin yielding methaemoglobin and NO_3^-(Liu et al., 1998). This reaction is so fast that all available NO would be consumed if barriers did not exist. Several diffusional barriers exist which are estimated to decrease the rate of NO scavenging by intra-erythrocytic Haemoglobin (Hb) greater than 600-fold:
    - Compartmentalisation of Hb within the erythrocyte.
    - Erythrocyte membrane and submembrane limiting the rate of NO reaction with Hb(Vaughn et al., 2000;Huang et al., 2001).
    - An unstirred diffusional barrier around erythrocytes(Liu et al., 1998;Coin and Olson, 1979).
    - Erythrocyte free zone of plasma along the surface of the vascular endothelium(Vaughn et al., 1998;Butler et al., 1998;Liao et al., 1999).
- *NO can be transported in blood and tissues in the form of RSNOs*, e.g. S-nitrosylation of albumin. NO can also bind to cysteine residues in Hb (SNO-Hb). S-nitrosated haemoglobin and albumin act as stable storage forms of intravascular NO and as an allosterically regulated delivery vehicle for NO(Jia et al., 1996;Stamler et al., 1992b). The main criticism to this theory is the lack of evidence for the existence of significant amounts of S-nitrosated proteins in the human circulation. Also, in contrast to nitrite, there are no detectable arterial-to-venous gradients of S-nitrosated Hb in the human circulation(Gladwin and Schechter, 2004).

- The most recent theory is that *NO is generated from NO_2^- by simple reduction*, this can occur in both blood and tissues(Cosby et al., 2003). Nitrite is regarded by many as an ideal vascular storage pool for NO, it is present at fairly high levels in blood and is more stable than NO/SNOs. With sufficient oxygen the predominant reaction product is NO_3^-, however NO is favoured when oxygen falls, creating an autoregulation system. The demonstration of artery-to-vein gradients of NO_2^- in the human circulation and increased consumption of NO_2^- during exercise stress, provide evidence to support NO_2^- as an NO transporter(Gladwin et al., 2000b). It is proposed that release of NO occurs following the reaction of deoxyHb ($HbFe^{II}$) with NO_2^-, vasodilation occurs as Hb unloads oxygen to 50% saturation. Xanthinoxidoreductase, MetHb and NO_3^- can also liberate NO. Nitrite fits the requirements for a physiological mediator of hypoxic vasodilation because it maximally reacts with Hb at 50% Hb saturation (P_{50} Hb). This oxygen tension is significantly higher than that required for SNO-Hb deoxygenation(Gladwin et al., 2006).

1.4. Biological roles of NO

1.4.1. General

NO is well known for its divergent effects, these effects of NO are dependent on a number of factors, including: the source of NO, the redox form and pH of the microenvironment, and the target cells in tissue. NO has an astounding range of biological roles including modulation of vascular tone, memory formation and inflammation(Moncada and Higgs, 1993;Southam and Garthwaite, 1993;Schmidt and Walter, 1994;Nussler and Billiar, 1993). NO often achieves these effects by binding to the haem group of the soluble form of the enzyme guanylate cyclase, but rarely through irreversible chemical modifications of other molecules. NO is a prime example of a molecule which may normally be useful but is toxic in excess.

The most studied actions of NO are in the cardiovascular system. Extremely low (pico-nanomolar) amounts of NO are continuously produced by eNOS in the endothelial cells that line the lumen of blood vessels. This homeostatic NO production is responsible for vasodilatation, vascular smooth muscle cell inhibition, inhibition of platelet aggregation and adhesion and inhibition of inflammatory cell activation and monocyte activity(Miller and Megson, 2007b).

The properties and cellular targets of NO at higher concentrations are markedly different to those of NO at lower concentrations. Under oxidative stress, NO rapidly reacts with superoxide to form $ONOO^-$. Under these circumstances NO is highly cytotoxic, a feature that is exploited by inflammatory cells in response to invading pathogens by expressing iNOS in concert with activation of NAD(P)H oxidase to generate NO and superoxide forming $ONOO^-$, which has a highly cytotoxic and cytostatic role (figure 1.1). At high concentrations, NO also reacts with molecular oxygen, generating nitrosating species capable of regulating protein and cell function(Gow et al., 2004). NO, endogenous RSNOs and $ONOO^-$ can have an inhibitory impact on cellular respiration through interaction with complexes in the respiratory chain(Beltran et al., 2000;Brown and Borutaite, 2004). High concentrations of NO and related species can also mediate apoptosis in inflammatory cells(Taylor et al., 2003).

1.4.2. NO and inflammation

The pleiotropic actions of NO are exemplified when studying the role it plays in inflammation. NO has many anti-inflammatory properties, such as the effects that it has on vascular permeability: NO diminishes endothelial permeability(Granger and Kubes, 1996) and NO donors have been found to reduce oedema formation in various experimental models(Hinder et al., 1999). NOS inhibitors can exacerbate oedema formation(Mundy and Dorrington, 2000). Leukocyte and platelet adherence to the endothelium are inhibited by NO(Kubes et al., 1991;Clancy et al., 1992), as is the production of superoxide anions by leukocytes(Clancy et al., 1992). In addition NO inhibits macrophage degranulation(Clancy and Abramson, 2000).

Evidence also exists for the pro-inflammatory properties of NO: in experimental models of inflammation it has been shown to promote carrageenin-induced oedema in the mouse footpad(Ianaro et al., 1994), it also induces vasodilation in the neurogenic inflammatory reaction of the rat hindpaw skin to topical application of mustard oil(Lippe et al., 1993).

1.4.3. NO in human inflammatory diseases

1.4.3.1.Lupus erythematosus

NO has been implicated in numerous inflammatory diseases. Weinberg *et al.*, demonstrated that the murine model of lupus (MRL/lpr mice) develop a spontaneous autoimmune disease resembling systemic lupus erythematosus (SLE). MRL/lpr mice excrete significantly higher concentrations of urinary NO_2^-/ NO_3^- as an indicator of increased NO generation(Weinberg et al., 1994). MRL/lpr mice fed a diet rich in L-arginine develop more severe nephritis, although $iNOS^{-/-}$ mice are not protected from the development of arthritis and nephritis(Peters et al., 2003). An upregulation of iNOS has been demonstrated in vascular endothelium from patients with SLE(Belmont et al., 1997). An increase in serum NO_2^- has been shown to be proportional to disease activity and double stranded DNA Ab titres in human SLE, the strongest correlation is between serum NO_2^- levels and renal disease(Peters et al., 2003).

1.4.3.2.Arthritis

iNOS expression has been shown to be upregulated in the synovium of both rheumatoid arthritis (RA) and osteo arthritis (OA) patients(Sakurai et al., 1995;McInnes et al., 1996;Grabowski et al., 1997;Armour et al., 1999). iNOS is localised mainly to fibroblasts in the synovial membrane of RA patients, evidence suggests that it upregulates tumour necrosis factor α (TNFα) which subsequently leads to the inflammation of RA(McInnes et al., 1996).

1.4.3.3.Systemic sclerosis

As with many diseases the role of NO in systemic sclerosis (SSc) may be seen as paradoxical. Impaired basal NO production may contribute greatly to the development of SSc vascular disease by enhancing vasospasm, platelet aggregation, upregulation of endothelial and leukocyte adhesion molecules and increasing vascular wall thickness. Deficient NO release from SSc vascular endothelium *in vitro* has been demonstrated (Freedman et al., 1999;Romero et al., 2000). In contrast, an increase in iNOS expression has been demonstrated in lesional skin of SSc patients(Yamamoto et al., 1998;Cotton et al., 1999), and an increase in circulating NO_3^- in SSc correlates with markers of endothelial damage and disease activity(Yamamoto et al., 1998). In SSc, endothelial injury seems to reduce eNOS and increase iNOS activity, resulting in a vasconstricting and proinflammatory environment in association with tissue damage(Matucci and Kahaleh, 2002).

1.4.3.4.Atherosclerosis

It is now widely recognised that there is an inflammatory component to the pathogenesis and progression of atherosclerosis(Ross, 1999). Recruitment of inflammatory cells is the major driving force behind plaque development. Apoptotic macrophages are present in atherosclerotic plaques in both human and animal disease models(Ludewig et al., 2002). iNOS has been demonstrated in human atherosclerotic plaques(Buttery et al., 1996). The process of resolution of inflammation involves the phagocytosis of apoptotic cells, thus preventing any further initiation of a proinflammatory response (figure 1.5). Apoptosis may represent a mechanism for atherosclerotic plaque regression. NO is a promising candidate for this pathway because, as well as having pro-apoptotic actions it has several other powerful antiatherogenic characteristics including a powerful inhibitory effect on platelet and inflammatory cell activation(Shaw et al., 2005). L-arginine administration to hypercholesterolaemic rabbits increases the number of apoptotic macrophages in intimal lesions three-fold, this is associated with plaque regression(Wang et al.,

1999). These observations suggest that manipulation of the NOS pathway may represent a therapeutic approach for atherosclerosis.

1.4.3.5.Asthma

The role of NO in asthma is of interest as recent evidence suggests more complex interactions than were initially observed. These observations are comparable to those made for psoriasis, in both diseases internal feedback control mechanisms, involving the activity of arginase (figure 1.4), determine tissue NO levels and pathogenesis. Initial observations revealed an increase in NO in the exhaled air of asthmatics, which was thought to be a marker of airway inflammation(Alving et al., 1993;Saleh et al., 1998). Conflicting data exists for the role of iNOS in a murine asthma model: one group suggest that acute inhibition of iNOS activity has no effect(Feder et al., 1997), whereas a second suggest inhibition of iNOS activity suppresses airway inflammation(Trifilieff et al., 2000), and finally a third group show that iNOS inhibition exacerbates airways inflammation(Blease et al., 2000).

Keller *et al.*, suggest a hypothesis for iNOS expression in experimental asthma which stems from the presence of endotoxin lipopolysaccharides (LPS) (figure 1.4). LPS is a cell wall component of gram-negative bacteria, which signals through toll-like receptor 4 (TLR4) and leads to up-regulation of iNOS activity(Wagner et al., 1983;Stuehr and Marletta, 1985). LPSs are ubiquitous in the environment and found in household dusts allergens and allergen extracts(Michel et al., 1991;Gereda et al., 2000). After allergen inhalation, two enzymatic pathways, iNOS and arginase 1, are activated. The arginase1 pathway is activated by type 2 cytokines released from allergen-driven T helper 2 (Th 2) cells, whereas LPS activate the iNOS pathway.

L-OH Arginine, a by product of the conversion of L-arginine by iNOS, inhibits arginase 1, whereas arginase 1 depletes L-arginine and generates polyamines that limit NO production and down regulate iNOS expression, respectively(Bronte et al., 2003;Meurs et al., 2003). Both pathways are activated and are reciprocally

downregulated, therefore it is likely that L-arginine concentrations are at low levels following allergen inhalation. It has been shown that iNOS-dependent catabolism of L-arginine at low levels favours the production of $ONOO^-$, known for its proinflammatory actions(Xia and Zweier, 1997;Xia et al., 1998). In addition low local levels of NO have been shown to promote the expression of proinflammatory proteins including COX2 and IL-6(Connelly et al., 2001).

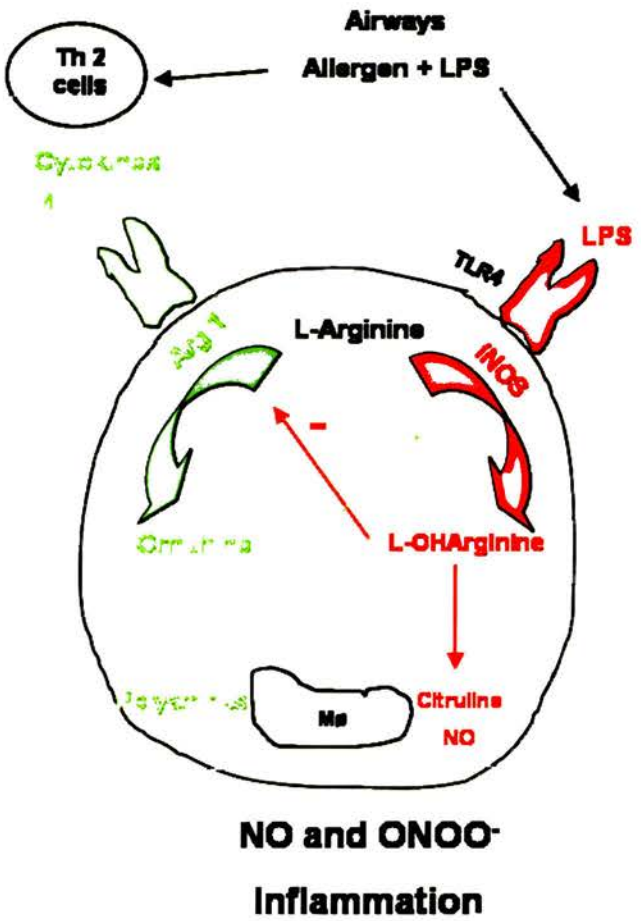


Figure 1.4 *Role of lipopolysaccharides, iNOS and arginase 1 in airway inflammation*

Adapted from(Keller et al., 2005)

1.4.4. NO and the resolution of inflammation

Recent studies have revealed that NO can modulate apoptosis in a variety of cell types, including human inflammatory cells(Taylor et al., 2003). Macrophages were

the first cell type in which NO mediated apoptosis was demonstrated(Sarih et al., 1993;Albina et al., 1993;von Knethen et al., 1999). In addition, a pro-apoptotic effect of NO has been demonstrated in neutrophils(Ward et al., 2000;Singhal et al., 1999;Fortenberry et al., 1999;Taylor et al., 2001) and eosinophils(Beauvais et al., 1995). NO has a bi-phasic effect on apoptosis in many cell types, this has been shown in neutrophils, where NO may also have an anti-apoptotic potential. Low concentrations of NO generated from the spontaneous NO donors, spermine/NO (SPER/NO) and diethylamine (DEA/NO), reduce the rate of neutrophil apoptosis(Taylor et al., 2003). The same study also demonstrated that a different NO donor, GEA-3162, at equivalent concentrations, produced no inhibition. GEA-3162 decomposes to generate NO and O_2^- which then forms $ONOO^-$, suggesting that the pro- or anti-apoptotic effects of NO may be governed by the specific NO-related species generated.

Apoptosis of inflammatory cells is a highly regulated process whereby cellular death occurs without the disruption of the cell membrane, and subsequent release of the pro-inflammatory and histotoxic contents of the dying cell(Haslett, 1997). Apoptotic cells are instantly recognised and ingested by phagocytes, such as macrophages, using mechanisms that down-regulate pro-inflammatory mediator release, and increase the release of agents with anti-inflammatory potential from the ingesting cell(Meagher et al., 1992;Fadok et al., 1998;Liu et al., 1999). Apoptosis represents a non-inflammatory mechanism to remove potentially damaging pro-inflammatory cells from the site of inflammation, it is critical to the successful resolution of the inflammatory response. Chronic inflammation is frequently characterised by a failure of myelocytic cells to undergo apoptosis, or of phagocytes to remove apoptotic cells. Evidence suggests that NO is particularly relevant in the resolution of inflammation. High concentrations of iNOS derived NO synthesised in macrophages (phagocytes), induce apoptosis in neighbouring cells. Apoptotic cells are subsequently recognised and ingested by phagocytes, thus promoting the resolution of inflammation. Conversely, low concentrations of NO produced constitutively by eNOS in endothelial cells can inhibit apoptosis(Taylor et al., 2003)(figure 1.5).

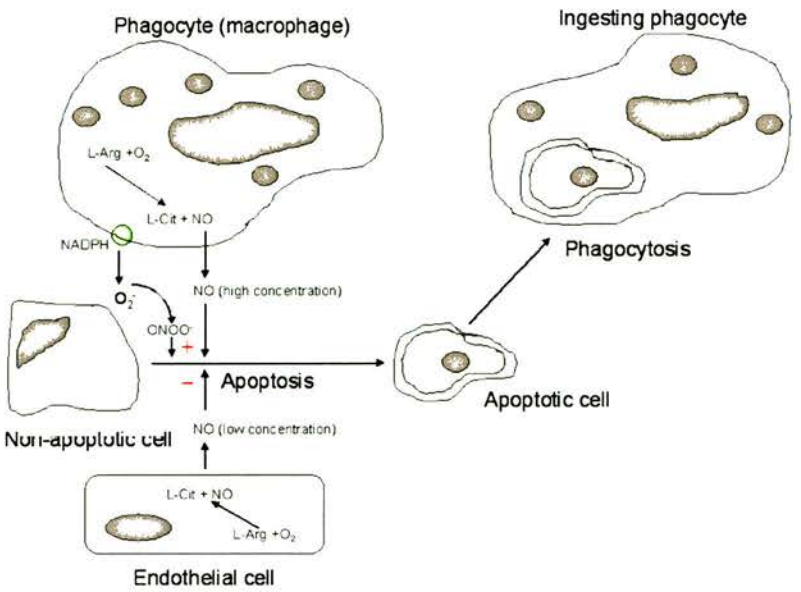


Figure 1.5 *NO and the resolution of inflammation*

Adapted from(Taylor et al., 2003)

1.4.5. NO in skin homeostasis

The skin vascular system is in a constant state of active regulation due to the generation of NO(Lowenstein and Snyder, 1992). Small pulses of NO are produced via eNOS activity in the endothelial cells of skin vessels, which results in smooth muscle relaxation(Anggard, 1994). It has been demonstrated that NO is involved in maintaining resting cutaneous blood flow, as the injection of intradermal eNOS antagonists impair local skin circulation(Goldsmith et al., 1996).

NO has been shown to play a role in cutaneous melanogenesis, evidence exists to show that NO synthesis by nNOS and subsequent cGMP production in melanocytes is required for ultraviolet B (UVB)-induced melanogenesis(Romero-Graillet et al., 1996). In addition NO is involved in the control of keratinocyte proliferation and differentiation(Krischel et al., 1998), and in the regulation of hair follicle activity(Wolf et al., 2003).

1.4.5.1.NO and responses to environmental challenges

NO plays a major role following ultra-violet radiation (UVR) in the development of erythema(Warren, 1994), oedema(Teixeira et al., 1993) and melanogenesis(Romero-Graillet et al., 1996;Romero-Graillet et al., 1997) (Ch1.6).

NO is responsible for forming a protective antimicrobial barrier on the skin surface. Nitrate present in sweat on the skin surface is reduced by bacteria to produce NO_2^-, which is then reduced in acidic conditions to produce NO(Weller et al., 1996). Several pathogens such as *Escherichia coli*, *Candida albicans* and mycobacteria have been shown to be sensitive to the cytotoxic effects of NO, its generation on the skin surface is thought to regulate the growth of skin commensal organisms and prevent infection with skin pathogens(Bruch-Gerharz et al., 1998).

1.4.6. NO in skin disease

With the advent of immunostaining for iNOS, iNOS protein has been demonstrated immunohistochemically in many inflammatory dermatoses including: psoriasis(Kolb-Bachofen et al., 1994;Sakai et al., 1996), atopic dermatitis(Rowe et al., 1997;Clancy et al., 1998) and lupus erythematosus (LE)(Clancy et al., 1998). The iNOS enzyme is consistently found in the dermis and basal epidermis of psoriatic plaques(Bruch-Gerharz et al., 1996;Rowe et al., 1997), and the characteristic erythema seen clinically, indicates vasodilatation and thus presumably biologically significant NO production.

1.4.6.1.Psoriasis

Measurements with a chemiluminescence meter from the surface of psoriatic plaques show elevated NO release(Ormerod et al., 1998), and serum NO_2^-, an indicator of systemic NO production, is elevated in psoriatics with active disease(Tekin et al., 2006). However, as is often the case with NO, the role it has in the pathogenesis of

psoriasis is more complex than can be explained by a simple increase in NO production.

iNOS is invariably expressed in keratinocytes from psoriatic skin lesions, this suggests an influence of NO on keratinocyte proliferation and differentiation. Such an effect is demonstrated by the maintenance of normal human keratinocytes in the presence of NO donors for prolonged periods of time, mimicking an increased local NO production(Krischel et al., 1998). Interestingly, NO has been found to exhibit a biphasic effect on keratinocyte proliferation and differentiation. At low NO donor concentrations a significant increase in keratinocyte proliferation was observed, whereas at higher concentrations – comparable to iNOS-mediated synthesis rates – induction of keratinocyte differentiation was detected, with no cytotoxic effect on the cells(Krischel et al., 1998). These findings demonstrate the involvement of NO in the regulation of epidermal growth and development. It is postulated that this biphasic role of NO in the control of keratinocyte proliferation and differentiation is active in psoriasis. iNOS and subsequently NO are elevated in psoriatic plaques, however arginase is also upregulated. Arginase competes with NOS for L-arginine which results in an elevation in NO that is sufficient to induce keratinocyte proliferation but not differentiation(Bruch-Gerharz et al., 2003).

1.4.6.2. Wound healing

The healing process requires an intricate interaction between inflammatory cells, biochemical mediators including growth factors and extracellular matrix molecules, and microenvironmental cell populations(Albina et al., 1990;Yamasaki et al., 1998). NO has been shown to play a major role in wound healing(Frank et al., 2002). Reduction in the levels of wound NO by gene knockout or pharmacological inhibition results in impairment of wound healing, as evidenced by increased closure time, lower wound breaking strength and decreased collagen deposition(Schaffer et al., 1999). Conversely, supplementation with L-arginine, increases the healing of wounds(Seifter et al., 1978). This augmentation of wound healing is not seen in NOS

knockout mice, further underlining the importance of wound NO synthesis from L-arginine(Shi et al., 2003). In addition, transfection of iNOS cDNA into wounds results in marked increases in collagen deposition(Thornton et al., 1998;Yamasaki et al., 1998). Recent evidence suggests that NO applied in the form of an acidified nitrite NO donor, can augment wound healing in normal and diabetic mice. However, the effects of this preparation are dependent both on the time of application after wound healing and the concentration of topical NO donor. Application of acidified nitrite immediately after wounding impairs healing, whereas application on day three after wounding results in significant healing(Weller and Finnen, 2006b).

1.4.6.3.Infectious skin disease

NO synthesised at high concentrations by iNOS, has been shown to eliminate intracellular pathogens including *Mycobacterium tuberculosis*, *M. Leprae*, *Leishmania* species, *Trypanosoma cruzi* and *Plasmodium falciparum* and also to block viral replication(Liew and Cox, 1991;Liew et al., 1991;Scharton-Kersten et al., 1997;Stenger et al., 1996). Most cell types express iNOS, however epithelial cells of the lung, the liver, the gastrointestinal tract and the skin, which act as immunological barriers, appear to use this mechanism as a first line of defence.

Weller *et al.*, provide data supporting an antimicrobial effect of NO in the skin. Acidified NO_2^- cream, which releases NO via the intermediate N_2O_3, was used successfully as a treatment for tinea pedis(Weller et al., 1998). More recently this topical treatment has also been successful in treating onychomycosis, > 90% of patients became culture negative for *Trychophyton rubrum* following treatment with acidified NO_2^-. S-nitrosothiols, formed by nitrosation of nail sulphur residues, were shown to be the active component(Finnen et al., 2007). Acidified NO_2^- exploits the nature of the nail barrier and utilizes it as a means of delivery of NO/nitrosothiol-mediated antifungal activity. Thus what is commonly regarded as the principal obstacle to therapy in the nail becomes an effective delivery mechanism.

1.4.6.4.Inflammatory or immune-mediated skin disease

It is thought that high-output NO synthesis is critical for immunological reactions in human skin. *In vitro* experiments have demonstrated that interleukin-8 (IL-8), interferon-γ (IFN-γ), TNF-α and interleukin-1β (IL-1β) are potent synergistic inducers for iNOS expression in human keratinocytes(Bruch-Gerharz et al., 1996). It is also hypothesised that aberrant upregulation of iNOS following UVR exposure, is involved in the pathogenesis of cutaneous lupus erythematosus (cLE)(Kuhn et al., 1998). The histologic changes of cLE include keratinocyte apoptosis and inflammation, two processes which are known to be influenced by NO.

It has been demonstrated that NO can be produced in Langerhans cells of human skin(Qureshi et al., 1996). It is thought that NO may affect Langerhans cell functions such as antigen presentation and cytotoxicity, and may also be used for the functional regulation of adjacent keratinocytes and melanocytes. Upregulation/induction of iNOS expression has been shown in murine Langerhans cells and keratinocytes during contact hypersensitivity reactions in mice(Ross et al., 1998).

iNOS expression has been shown in dermal microvascular endothelial cells. In inflammatory processes, the regulation of leukocyte trafficking involves a complex interplay of adhesion molecules and chemokines, with cytokines produced by both leukocytes and endothelial cells(Baggiolini, 1998;Suschek et al., 1993). Evidence indicates that iNOS expression and concomitant increases in NO production in endothelial cells modulate lymphocyte adhesion as well as leukocyte trafficking(Khan et al., 1996).

NO may regulate important humoral and cellular responses in innate immune and inflammatory processes throughout the skin. NO may also be implicated in the pathogenesis of various inflammatory and immune-mediated skin diseases. It is

known that NO can exert both pro-inflammatory and anti-inflammatory properties, even in parallel, depending on the cellular context, and the type and phase of the inflammatory and cellular immune response(Kolb and Kolb-Bachofen, 1998b;Kroncke et al., 1997). *In vivo* research in human subjects is vital if we are to advance our understanding of the roles of NO in inflammatory processes. The human skin is relatively accessible, which makes it an ideal organ in which to further elucidate the role of NO in these processes in both normal and disease states.

1.5. NO stores

1.5.1. Evidence of biological activity

The historical view of NO has been that it can only act in a paracrine manner on neighbouring cells because of its very short half life. However, more recent studies have suggested that the bioactivity of NO in blood in fact can be conserved, thereby allowing for more distal and sustained effects:

- SNO-Hb acts as a form of bioactive NO(Jia et al., 1996).
- NitrosylHb and NO_2^- act as potential sources of NO(Gladwin et al., 2000a).
- NO itself may remain active in the blood stream longer than originally assumed(Rassaf et al., 2002c).
- RSNO derivatives of plasma proteins may conserve/transport NO(Rassaf et al., 2002c;Rassaf et al., 2002b;Stamler et al., 1992a).

Conservation of the bioactivity of NO is enabled by the formation of NO-related products, these storage forms of NO include: NO_3^-, NO_2^-, RSNOs and RNNOs. Lundberg *et al.*, have shown that following ingestion of a NO_3^--rich meal (10mg/kg sodium NO_3) there is a rise in saliva, plasma and urine NO_3^- concentrations, with subsequent increases in salivary and plasma RSNO, and plasma NO_2^- concentrations(Lundberg and Govoni, 2004).

1.5.2. Individual NO-related products

1.5.2.1.Nitrate (NO_3^-)

Plasma NO_3^- levels are determined by dietary intake of NO_3^- and by endogenous NO production. Diet is the main source of NO_3^-, vegetables form 60-80% of the daily NO_3^- intake of a typical western diet(Spiegelhalder et al., 1976). The main endogenous source of NO_3^- is the L-arginine-NO pathway. An entero-salivary recirculation pathway exists for NO_3^-, 25% of all circulating NO_3^- is taken up by the salivary glands and secreted in saliva, the resulting salivary NO_3^- concentration is ten times higher than that in plasma. (Lundberg et al., 2004) (figure 1.6). Ingested NO_3^- is absorbed proximally from the gastrointestinal tract into the blood stream where it mixes with endogenously synthesised NO_3^-. Peak plasma concentrations are seen within 60 minutes of NO_3^- ingestion(Lundberg and Govoni, 2004), the $t_{1/2}$ of NO_3^- in plasma is in the order of 5 - 7 hours(McKnight et al., 1997). It was thought for a long time that human cells can not metabolise NO_3^-, however recent evidence has shown that a mammalian functional nitrate reductase does exist(Jansson et al., 2008). Oral commensal bacteria reduce salivary NO_3^- to NO_2^-, on entering the acidic environment of the stomach NO_2^- reacts with stomach acid releasing NO(Benjamin et al., 1994;Modin et al., 2001). Reduction of NO_2^- can also occur: in the oral cavity(Duncan et al., 1995), on skin(Weller et al., 1996), in urine(Lundberg et al., 1997) and systemically in blood and tissues.

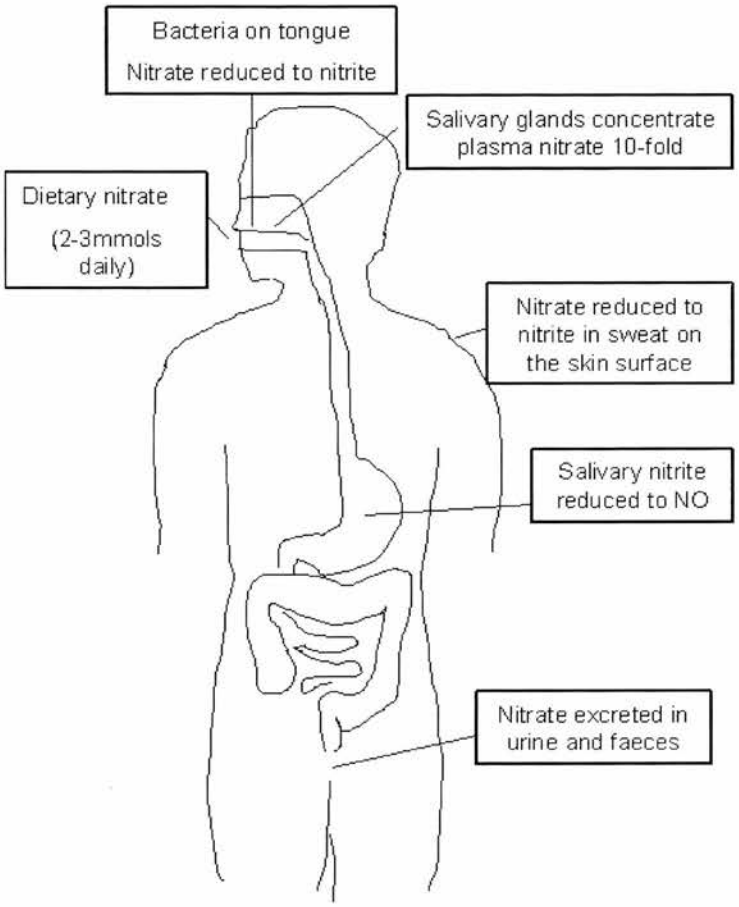


Figure 1.6 *The entero-salivary circulation of nitrate*

Adapted from(Lundberg et al., 2004)

1.5.2.2.Nitrite (NO_2^-)

There are three sources of NO_2^- in mammals:

- An oxidation product of NOS.
- Dietary, NO_2^- is found as a food additive in meat to prevent botulism and to enhance its appearance(Lundberg et al., 2004).
- Generated from commensal bacteria in the digestive system by NO_3^- reduction.

The majority (80-90%) of plasma NO_2^- originates from eNOS activity, therefore it is often regarded as a biomarker for eNOS function(Kleinbongard et al., 2003).

There is a relatively wide range of reported human basal plasma NO_2^- concentrations, from 75nM to 26μM(Pelletier et al., 2006;Gorenflo et al., 2001) (table 1.1). Explanations for such a wide range in plasma NO_2^- include: the rapid metabolism of NO_2^- to NO_3^-, variations in blood sampling and sample processing, and methodological problems in analysis (some methods are not sensitive enough, analysis may also be affected by proteins, varying redox conditions, contamination with Hb and tracc contamination with NO_2^- during processing).

Plasma NO_2^- levels are higher than those of RSNOs. It was initially suggested that RSNOs are the main carriers of NO in the circulation, however there is increasing evidence that NO_2^- is the more important NO-related product (Lundberg et al., 2004). A number of groups have published evidence which suggest that NO_2^- is a major intravascular storage pool for NO:

- NO_2^- is present in relatively high concentrations in plasma(Kleinbongard et al., 2003) and tissue(Rodriguez et al., 2003).
- NO_2^- is relatively stable as it is not reduced by cellular reductants that affect RSNOs and NO(Gladwin et al., 2002).
- The reaction rate of NO_2^- with haem proteins is 10,000 times less than that of NO(Dejam et al., 2004).

During conditions of ischaemia and acidosis, NO_2^- can release NO by non-enzymatic mechanisms(Zweier et al., 1995):

- NO_2^- can form nitrous acid, which can react with NO_2^- again or an electron donor (such as ascorbate) to form N_2O_3. Dinitrogen trioxide can then nitrosate thiols or, in the presence of an electron donor, produce NO gas(Wink et al., 1996a).
- Xanthinoxoreductase, which is abundant in vascular tissue, can reduce NO_2^- to NO(Millar et al., 1998).

Nitrite has been shown to act as a signalling molecule in its own right(Bryan et al., 2005). Physiological data also suggests that NO_2^- has direct effects, inhaled NO can have vasodilatory effects not only locally in the pulmonary circulation but also in peripheral tissues, this is associated with an increase in NO_2^-(Cannon, III et al., 2001;Fox-Robichaud et al., 1998;Takahashi et al., 1998).

1.5.2.3.Nitrosothiols (RSNOs)

NO can react with a number of molecular targets in the blood following reaction with molecular oxygen or reactive oxygen species to form NO_2, N_2O_3, or $ONOO^-$. Unlike NO, these species are nitrosating or nitrating agents that can react with aromatic compounds, amines, alcohols and thiols to form C-, N-, O- and S-nitroso species(Rassaf et al., 2002c). Products of NO oxidation (NOx) rapidly react with SH-groups, e.g. in proteins, to form RSNOs like: S-nitrosoalbumin, S-nitrosoglutathione, or S-nitroso-L-cysteine. Albumin, the principle plasma protein, reacts with NO to form S-nitrosoalbumin (SNOAlb), this confers NO^+ (nitrosonium), NO^- (nitroxyl), and NO-donating properties to this molecule(Rassaf et al., 2002a). Human albumin contains a single free SH-group in Cys-34, which binds to NO(Kashiba-Iwatsuki et al., 1997).

There is no consensus as to the true physiological levels of RSNOs, published data suggests levels in human plasma ranging from 6.3nM to 7μM(Lundberg and Govoni, 2004;Stamler et al., 1992a) (table 1.1). There is general agreement that NO_2^- is present in human plasma in greater levels than RSNOs. Similarly to the measurement of NO_2^-, the variation in published RSNO concentrations may have their origin in the different methodological approaches used and associated technical difficulties, including artifactual RSNO formation, inherent to trace level analysis of NO-related products.

The biological effects of RSNOs resemble NO but their $t_{1/2}$ is much longer, effects include: vasodilatation(Keaney, Jr. et al., 1993), smooth muscle relaxation(Iversen et

al., 1994;Jansen et al., 1992), and inhibition of platelet aggregation(Radomski et al., 1992;Simon et al., 1993).

1.5.2.4.N-nitrosamines (RNNOs)

Little is known about the reaction sites of NO other than thiols. It is known that RNNOs are generated endogenously during infections and inflammatory processes(Ohshima and Bartsch, 1994). In the acidic environment of the stomach, RNNOs are formed due to the reaction of NO_2^- with amino groups of food constituents(Lijinsky, 1980) and secondary amines. Irrespective of whether they are taken up during occupational exposure, ingested with the diet(Lijinsky, 1999), or formed endogenously, most low molecular weight RNNOs are potentially mutagenic and have been associated with an increased risk of cancer(Ohshima and Bartsch, 1994).

1.5.3. Quantification of NO-related products

Many different methods to measure plasma NO-related products have been proposed, due to the rapid metabolism of NO_2^- in blood and the difficulties in its analytical determination, an agreement on which is the most reliable method has not yet been reached. The result of such disagreement is a variation in published data from different groups regarding the concentration of NO-related products in human biological samples (table 1.1).

Existing methods for the quantification of NO-related products include:

- The Griess assay – a colorimetric method used to quantify NO_2^-, it was first described by Peter Griess in 1858. Sulphanilic acid is added to a NO_2^- containing sample forming a diazonium salt. An azo dye is then added (N-1-napthylethylenediamine HCL) which couples with the diazonium salt to form an azo dye with a red pink colour(Griess, 1879).

- The Saville assay – this was originally described for the quantification of thiols, it is a technique based on mercuric chloride ($HgCl_2$) – induced cleavage of RSNOs to release NO_2^-, which is then quantified using the Griess assay (Saville, 1958).
- Electron paramagnetic resonance (EPR) spectroscopy – has been shown to be useful for determination of NO in metal complexes or haem proteins(Henry and Guissani, 2000). It can be applied *in vivo*, however it does not detect any other NO-related products and it has a low sensitivity.
- A more recent approach to NO-related products in biological systems involves liberation of molecular NO from the medium, followed by its detection via chemiluminescence accompanying its gas phase reaction with ozone. There are two classes of assays:
    - Those employing UV light to liberate NO photolytically. To distinguish FeNOs from SNOs, inorganic or organic mercury (Hg) is added. Mercury displaces NO from thiols in the form of NO_2^-. Proteins are then desalted in physiological buffers to remove low-molecular-weight reactants. The quantitiy of SNOs is calculated by measuring the loss of signal caused by Hg. All SNOs are reactive to Hg, whereas FeNO is unreactive, therefore the signal loss equates to the RSNO concentration(McMahon and Stamler, 1999).
    - Those employing chemical reactions. Identification depends on the differential reaction of NO-compounds toward a series of chemical reagents. Triiodide methodology is popular as it enables measurement of NO_2^- and RNNOs as well as SNO and FeNO(Feelisch et al., 2002).

Advocates of photolysis suggest the typically lower reported metabolite values measured using triiodide and other chemical based methods is the result of acid induced protein damage, and a general lack of understanding as to the chemical interactions in the mix. It is suggested that triiodide assays are strongly influenced by sample composition, rather than solely by the identity or quantity of NO species(Hausladen et al., 2007). In contrast, those groups favouring chemical assays argue that harsh photolytic exposure and secondary heating cause a lack of

specificity for RSNOs, and in biological samples thiols enhance NO release from NO_3^- photolysis leading to an overestimation of biological RSNO levels(Dejam et al., 2003). It is suggested that photolysis not only frees NO from RSNOs but also from compounds such as NO_2^-, nitrosamine and dinitrosyliron complexes(Rossi et al., 2001).

The originally documented RSNO concentration in human plasma was 7μMol/L, this was made by Stamler *et al.* using a photolytic method of analysis(Stamler et al., 1992a). The majority of recent studies have disputed these values and suggest levels in the nmol/L range (table 1.1). Kleinbogard *et al.* compare three different analytical methods of measuring plasma NO_2^-, the uniform presence of nanomolar concentrations (100-600nmol/l) of plasma NO_2^- was demonstrated in 90% of all individuals of various mammals including humans(Kleinbongard et al., 2003). The three methods of analysis compared were:

- Flow injection analysis combined with the Griess assay.
- Reductive gas phase chemiluminescence detection.
- High-pressure liquid chromatography combined with the Griess assay.

I have elected to use a gas phase chemiluminescence based assay for the quantification of NO-related products in biological samples, this method has been selected in view of the arguments presented above, and in particular because:

- There has been recent validation of this method for the quantification of NO-related products in biological samples(Feelisch et al., 2002).
- There is favourable comparison of results obtained using this method with those in the majority of published data.
- The sensitivity of this method for the detection of trace quantities of NO (femtomols), making it the method of choice for quantification of low levels of NO-related products. This is a factor which is of particular importance when analysing biological samples, as the yield of sample is often low (1-10μl).

- This method enables quantification of individual NO-related products: NO_2^-, RSNOs, RNNOs.

1.5.3.1.Gas-phase chemiluminescence detection of NO-related products

The concentration of NO_2^- and various NO-related products is determined after reductive cleavage by an iodide/triiodide-containing reduction mixture, and subsequent determination of the NO released into the gas phase by its chemiluminescent reaction with ozone (O_3). NO reacts with O_3 to form nitrogen dioxide (NO_2), a proportion of the NO_2 arises in an excited state (NO_2*). On decay to its ground state NO_2* emits light in the near infra-red region, which can be quantified by a photomultiplier(Clough and Thrush, 1967) (figure 1.7). The intensity of light emitted is directly proportional to NO concentration(Feelisch et al., 2002). NO concentration of biological samples is determined by comparing the output from the sample against a standard curve created from NO_2^- standards.

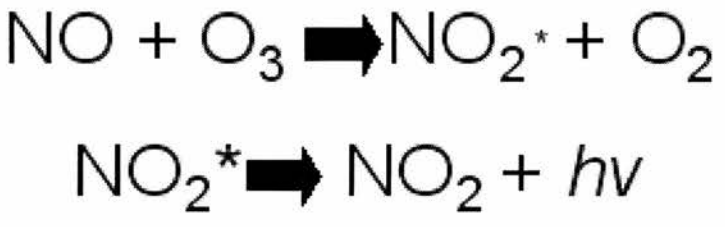


Figure 1.7 *Gas-phase chemiluminescence reaction of NO with ozone*

Quantification of individual NO-related products - Individual nitrosospecies can be quantified without having to change the reduction mixture or experimental conditions, this is achieved by pretreating samples with group-specific reagents before analysis (figure 1.8). A biological sample is divided into four aliquots:

- Direct injection into reduction mix (quantifies NO_2^- + RSNOs + RNNOs).
- Pretreatment with sulfanilamide (quantifies RSNOs + RNNOs).
- Pretreatment with $HgCl_2$/sulfanilamide (quantifies RNNOs + mercury resistant NO-related products).
- Pretreatment with NO_3^- reductase (quantifies NO_3^- + NO_2^- + RSNOs + RNNOs).

Nitrate is stable and not reduced by the iodide/triiodide-containing reduction mixture. The biological sample is pretreated with a NO_3^- reductase mix. The NO_3^- concentration is then determined by subtraction of the peak areas of untreated sample aliquots from sample aliquots pretreated with NO_3^- reductase mix.

Nitrite concentration of a biological sample is determined by subtraction of the peak areas of sample aliquots pretreated with sulfanilamide from that of untreated aliquots. Sulfanilamide reacts with the NO_2^- in the solution to form a stable diazonium ion.

S-Nitrosothiol concentration present in a biological sample is quantified by the subtraction of the peak areas of sample aliquots pretreated with $HgCl_2$/sulfanilamide from that of sample aliquots pretreated with sulfanilamide. Incubation with $HgCl_2$ causes cleavage of the S-NO bond without affecting peak shape or recovery of NO_2^- or NO, this reaction forms the basis of the Saville assay(Saville, 1958).

Mercury-resistant NO-related products, which include RNNOs or metal nitrosyls, are represented by the peak remaining after incubation with $HgCl_2$/sulfanilamide.

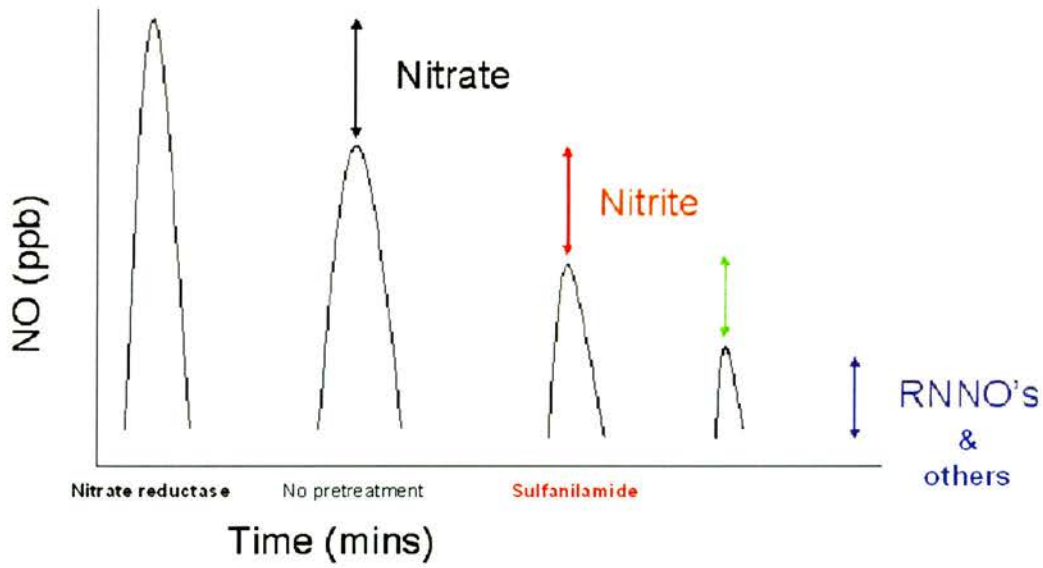


Figure 1.8 *Pretreatment of biological sample and quantification of individual NO-related products*

Author	Date	Method of analysis	Nitrate	Nitrite	RSNOs
Pelletier M(Pelletier et al., 2006)	2006	CLD	-	(plasma) 75-167nmol/L	-
Paunel AN(Paunel et al., 2005)	2005	CLD	(skin homogenate) 82.4±33.6μM	(skin homogenate) 5.1±1.6μM	(skin homogenate) 2.6±1μM
Lundberg JO(Lundberg and Govoni, 2004)	2004	CLD	(plasma) 30±4 μM (saliva) 0.19±0.03mM (urine) 0.8±0.3mM	(plasma) 123±19nM (saliva) 104±21 μM (urine) 3.4±1 μM	(plasma) 6.3±1.4nM (saliva) 25±9.8nM
Kleinbogard P(Kleinbongard et al., 2003)	2003	Similar results with: CLD, flow injection analysis, HPLC	-	(plasma) 305±23nmol/L	-
Rassaf T(Rassaf et al., 2002a)	2002	CLD	(plasma) 14.4±1.7μmol/L	(plasma) 0.20±0.02μmol/L	(plasma) 7.2±1.1nmol/L
Rossi R(Rossi et al., 2001)	2001	HPLC	-	-	(plasma) 30-40nmol/L
Tyurin VA(Tyurin et al., 2001)	2001	Photolysis	-	-	(plasma) 9.2±1.6nmol/L
Gorenflo M(Gorenflo et al., 2001)	2001	-	(plasma) 26.8μmol/L	(plasma) 26μmol/L	-
Cannon RO(Cannon, III et al., 2001)	2001	CLD	-	-	(plasma) 24±9nM
Moriel P(Moriel et al., 2001)	2001	CLD	(plasma) 19.1±12.03μM	(plasma) 1.01±0.53μM	(plasma) 0.25±0.2μM
Gladwin MT(Gladwin et al., 2000c)	2000	CLD	-	-	(plasma) undetectable
Marley R(Marley et al., 2000)	2000	CLD	-	-	(plasma) 28±7nMol/L
Goldman RK(Goldman et al., 1998)	1998	HPLC	-	-	(plasma) 220nmol/L
Fang K(Fang et al., 1998)	1998	Photolysis/CLD	-	-	(plasma) 930±360nM

Naseem KM(Naseem et al., 1996)	1996	Gradation of platelet inhibition	-	-	(plasma) 15-25nM
Weller R(Weller et al., 1996)	1996	Griess reagent	(sweat) 39.7±4.3μmol/L	(sweat) 3.42±0.4μmol/L	-
Benjamin N(Benjamin et al., 1994)	1994	-	-	(saliva) 114μM	-
Leone AM(Leone et al., 1994)	1994	HP capillary electrophoresis	-	(plasma) 450nmol/L	
Stamler JS(Stamler et al., 1992a)	1992	Photolysis		-	(plasma) 7μmol/L

Table 1.1. *Quantification of NO-related products in human biological samples*

1.6. UVR

Ultraviolet radiation is part of the electromagnetic spectrum (figure 1.9). In 1801 Johann Ritter discovered the UV region of the solar spectrum by showing that chemical action was caused by some form of energy in the dark portion beyond the violet(Ritter, 1801). In the previous year, Sir William Herschel had demonstrated the existence of radiation beyond the red end of the visible spectrum, a component now known as infrared radiation(Herschel, 1800). Ultraviolet, visible and infrared radiation, are referred to collectively as optical radiation.

Ultraviolet radiation spans the wavelength region from 400 to 100nm, the UVR spectrum is further subdivided into three regions: UVA, UVB and UVC. The idea of dividing the UV spectrum into spectral regions was first put forward at the Copenhagen meeting of the Second International Congress on Light, August 1932(Diffey, 2002). The three spectral regions were defined as:

- UVA 400-315nm
- UVB 315-280nm
- UVC 280-100nm

These divisions are arbitrary and differ depending on the discipline involved. Environmental and dermatological photobiologists normally define the wavelength regions as:

- UVA 400-320nm
    - UVA1 340-400nm
    - UVA2 320-340nm
- UVB 320-290nm
- UVC 290-200nm

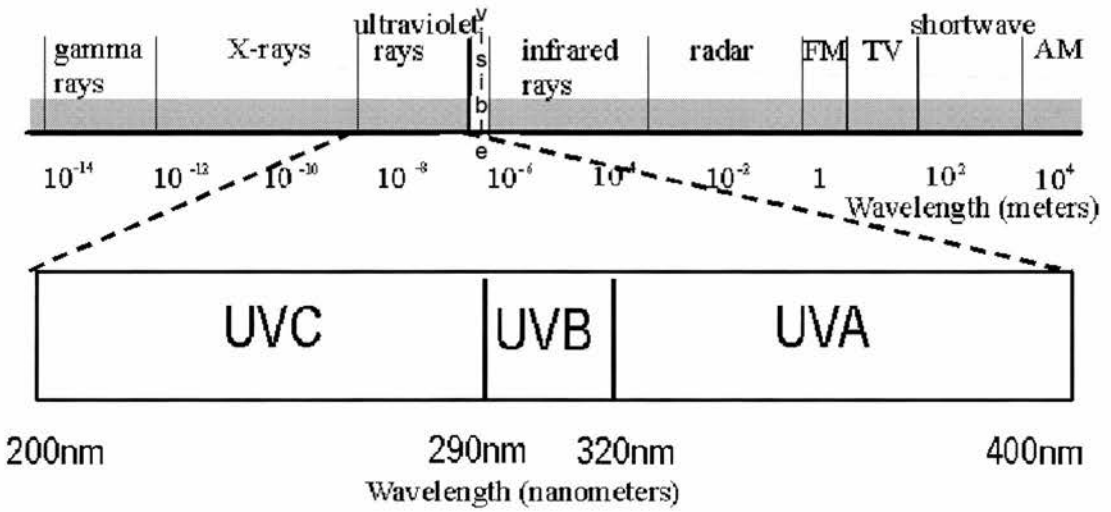


Figure 1.9 *UVR within the electromagnetic spectrum*

The quality (spectrum) and quantity (intensity) of terrestrial UVR vary with the elevation of the sun above the horizon. The elevation of the sun depends on the time of day, day of year, and geographical location (latitude and longitude). The quality and quantity of solar UV are modified as the sun's rays pass through the atmosphere. In the stratosphere (~10-15km above sea level) there is absorption by O_3 and scattering by molecules. In the troposphere (0~10km above sea level) there is absorption by pollutants and scattering by particulates and clouds. The attenuation of UVR in the atmosphere absorbs UV in a wavelength dependent manner, for example on a summer's day UVB constitutes 6% of the UVR reaching the earth's surface, the remaining 94% is UVA, UVC is absorbed by O_3 and does not reach the surface of the earth. Although UVB constitutes the smallest proportion of terrestrial UV when compared with UVA, it is the most effective at inducing erythema.

1.6.1. Radiometric calculations

The term 'irradiance' relates to the object (human subject) struck by the radiation, it is expressed as power (W) per m^2, the irradiance of a UV source is measured using a radiometer. The time integral of the irradiance is termed the 'radiant exposure', but is often expressed as 'dose'. The relationship between time, dose and irradiance is expressed in figure 1.10.

$$\text{Exposure time (min)} = \frac{1000 \times \text{prescribed dose (J cm}^{-2})}{60 \times \text{measured irradiance (mW cm}^{-2})}$$

Figure 1.10 *Radiometric calculations*

1.6.2. UVR and human skin

The skin is a primary target for UVR. UVR induces several cutaneous effects including: erythema(Rhodes et al., 2001), immune suppression(Damian et al., 2001), keratinocyte apoptosis(DANIELS, Jr. et al., 1961), accumulation of stable p53, DNA damage(Brash et al., 1991;Douki et al., 2003;Kobayashi et al., 2001;Mouret et al., 2006;Sheehan et al., 2002), skin cancer(Brash et al., 1991;Brash et al., 1996;Dumaz et al., 1993;Ziegler et al., 1993) and premature ageing(Gilchrest et al., 1979;Kripke, 1974).

1.6.2.1.Erythema

Erythema formation post UVR is a result of local increases in blood flow in both the superficial and deep vascular plexus of the dermis(Greaves, 1986). Erythema first becomes detectable 3-4 hours after exposure, is maximal between 8 and 24 hours, and persists for more than 48 hours(Anderson et al., 1991). The abilitiy of UVR to elicit erythema in human skin depends strongly on wavelength. A statement that a subject received an exposure dose of 1J cm^{-2} of UVR conveys no information regarding the likely erythemal effect. Studies show that UVB is orders of magnitude more effective per unit dose (J m^{-2}) than UVA at generating erythema. For example, the median 'minimal erythemal dose' (MED) at 300nm is 0.025J cm^{-2} whereas at 360nm it is 32J cm^{-2}(Young et al., 1998).

1.6.2.2.Measurement of erythema

For many years the term MED has been used as a 'measure' of erythemal radiation. The MED is judged by eye 24 hours after a predetermined UVR dose series has been

given. The MED is defined as the lowest UVR that will cause either a just perceptible redness or redness with a definite border. It is important to remember that this is a biological measure which is different for each individual, in view of this it has been suggested that MED should be reserved for use in observational studies in humans and other animals only.

More recently the term 'standard erythemal dose' (SED) has been proposed, this refers to erythemal effective radiant exposures from natural and artificial sources of UVR. One SED is equivalent to an erythemal effective radiant exposure of 100J m^{-2}(Diffey, 2002). Examples of the use of SED are:

- The ambient diurnal exposure on a clear sky summer day in Europe is approximately 30-40 SED.
- Four SED would be expected to produce moderate erythema on unexposed white skin, but little or no erythema on previously exposed white skin.

It is estimated that in skin types I-IV, one SED equates to 1.5-6 MEDs(Diffey et al., 1997).

1.6.2.3.Apoptosis

The term apoptosis comes from combining the Greek prefix ***apo-***, meaning 'off'or 'from' and ***ptosi***, meaning 'falling upon or in something', the entire word roughly translates to 'a falling off of leaves from a tree'. Apoptosis was coined by Kerr *et al.*, to differentiate between programmed cell death and necrosis(Kerr et al., 1972). There are two types of cell death:

- Apoptosis, an active process of single cell suicide, these cells are then phagocytosed by macrophages thus avoiding inflammation.
- Necrosis is induced by severe external insult, this affects groups of cells and usually induces an inflammatory reaction.

UVR induces apoptosis in human epidermal keratinocytes, keratinocytes undergoing apoptosis are termed 'sunburn cells'. Sunburn cells were first described by Daniels *et al.,* they are easily identified morphologically in haematoxylin and eosin (H&E) sections as being isolated epidermal cells with pyknotic nuclei, and shrunken eosinophilic cytoplasm(DANIELS, Jr. et al., 1961). Their presence suggests that cellular DNA has been irreparably damaged, it is generally accepted that their role is to remove UV-initiated potentially mutagenic cells from the skin(Claerhout et al., 2006). The major action spectrum for inducing sunburn cells is within the UVB range (290-320nm), they can also be found after irradiation with UVC (200-290nm) or high dose UVA (320-400nm). Sunburn cells are detectable from 8 hours after irradiation, maximal 24-48 hours and disappear by 60-72 hours(Kulms and Schwarz, 2000).

The three most important mechanisms for the induction of apoptosis are(Murphy et al., 2001):

- DNA photodamage, involving p53 induction – the two main lesions resulting from photochemical reactions within DNA are cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs), these are collectively termed DNA photoproducts. p53 causes cell cycle G1 arrest and regulation of transcription and cell differentiation. p53 dependent post-UVR arrest of cells in G1 allows DNA repair prior to DNA synthesis, this reduces DNA photoproducts and the need for apoptosis.
- Death receptor activation or release of their ligands – cell surface death receptors induce apoptosis on activation. The TNF death receptor superfamily includes: TNF-receptor-1, CD95 (Fas/Apo-1), TRAIL-receptors1 and 2, and death receptor-3 (DR3). UVA, B and C have all been shown to induce CD95 cxpression. Although DNA is the major molecular target for UVB, membrane/cytoplasmic targets, such as death receptors, are also relevant.
- Oxidative stress activates mitochondrial pathways and cytochrome C release – cytochrome C release seems to be an early and critical event in UVR-driven apoptosis. Apoptosis is normally finely regulated by pro- and anti-apoptotic

proteins. Anti-apoptotic proteins stabilise the transmembrane potential of mitochondrial membranes. UVR can initiate cell death by inducing 'megapore' formation in the mitochondrial membrane, this results in cytochrome C release into the cytoplasm which activates caspase 9 and thereafter apoptosis.

The terminal events in all apoptotic pathways include the activiation of a series of cytoplasmic proteases termed caspases.

Apoptosis combines a unique series of events, this was initially based on recognition of morphological changes in cell structures during apoptosis. Apoptosis is a highly conserved mechanism that shows uniformity through evolution, this allows extrapolation between species, systems, cell culture and intact tissue. The surface morphological features of apoptotic cells in culture include, blebs, echinoid spikes and surface blisters(Collins et al., 1997b) (figure 1.11).

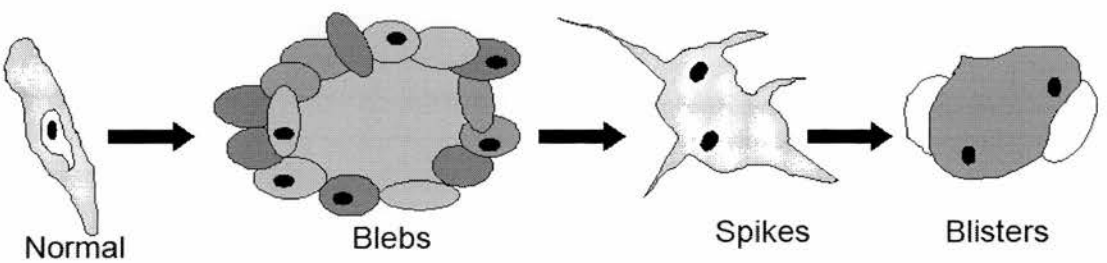


Figure 1.11 *Surface morphological changes during apoptosis*

Different events within the apoptosis process have been targeted in order to detect apoptotic cells in individual cells, mass cell cultures and intact tissues. The main categories of cellular changes that form the basis of apoptotic assays are seen below (subheadings highlight those methods that are appropriate for use in intact tissues, i.e. as in our studies):

- Changes in surface morphology and composition
    - Morphological changes evident on H&E (eg sunburn cells)
- Nuclear events and DNA cleavage
    - Segmentation of chromatin and nuclei
    - DNA cleavage in situ; detection of strand breaks
        - In situ nick-translation (ISNT)
        - Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL)
        - Anti-single-stranded DNA antibody
- Cell dissolution
- Cytoplasmic biochemical activation events
    - Caspase cleavage products
    - Caspase activity
- Mitochondrial function and integrity

During experimental design it is important to remember that apoptosis is not a synchronous event. Cells within a population may begin cell death at different times, and the lengths of the various stages of apoptotic morphological change can vary from cell to cell(Collins et al., 1997a). Some assays detect changes that occur early in the process whereas others detect changes that occur very late. In addition not all methods of detection are suitable for all cells undergoing apoptosis, for example Annexin V binding (a marker of phospholipid externalisation) has been shown only to occur in 30% of the cells undergoing apoptosis(Willingham, 1999). The asynchronicity of apoptosis, combined with the variability between different detection methods, dictates that when designing experiments to quantify apoptosis at

least two, if not three, different methods of detection should be employed. The most specific assay is probably the oldest, the detection of nuclear shape changes in early apoptosis.

1.6.2.4.DNA damage/repair

The type of DNA damage induced by UVR depends on the wavelength of the photons that hit the cell. UVB exposure results in direct light absorption by DNA, this induces dimerisation reactions between adjacent pyrimidine bases and the formation of DNA photoproducts (PPs). DNA PPs are characterised by C to T or CC to TT transitions. The transitions are regarded as 'signature' mutations, their presence is very suggestive of UV being the cause(Brash et al., 1991). Cyclobutane pyrimidine dimers in general and thymine dimers in particular, are the major DNA PPs. The remainder of PPs are accounted for by 6-4PPs. 6-4 photoproducts may be converted into their Dewar isomers upon exposure to near UVR (figure 1.12).

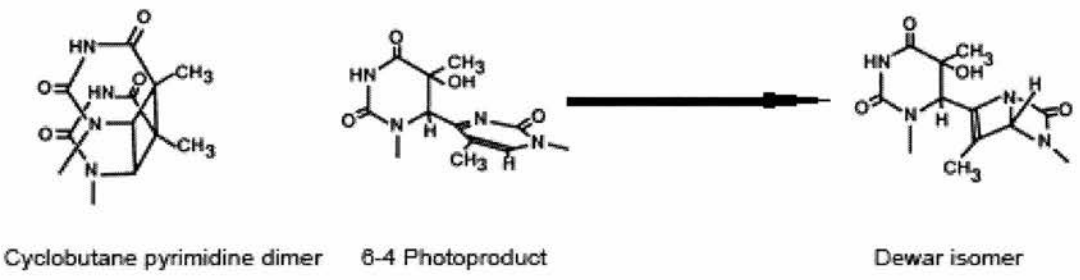


Figure 1.12 *DNA photoproducts*

UVB only contributes to 5% of UVR reaching the earth's surface, but it induces the majority of DNA damage due to the strong absorption of DNA at UVB wavelengths(Douki et al., 2003). However, all UVR wavelengths have been shown to induce DNA PPs(Douki et al., 2003;Kobayashi et al., 2001;Mouret et al., 2006;Sheehan et al., 2002).

The formation of 6-4PPs induces greater distortion in the DNA back bone than CPDs, this is thought to result in the comparably faster DNA repair of 6-4PPs that has been observed(Young et al., 1996). The actual rate of repair of UV induced DNA damage is widely debated and published figures for a $t_{1/2}$ of repair of CPD PPs ranges from 40-50% removal within 1 hour(D'Ambrosio et al., 1981;Reusch et al., 1988) to a $t_{1/2}$ of 33.3 hours(Young et al., 1996), with one group suggesting that almost complete removal of CPDs can take up to 72 hours after exposure to as little as 1.2MED(de Winter et al., 2001).

1.6.2.5.p53

Critical proteins involved in the response to DNA damage include the p53 tumour suppressor protein. p53 is an important transcriptional factor and also directly reacts with proteins such as nucleotide excision repair (NER)-associated regulatory proteins. Upon activation of p53, cells have two possible outcomes(Chow and Tron, 2005):

- Cell cycle arrest with DNA repair
- Apoptosis

Evidence for the involvement of PPs in skin carcinogenesis is provided by the finding of C to T and CC to TT tandem mutations in p53 tumour suppressor genes isolated from skin tumours(Brash et al., 1991;Brash et al., 1996;Dumaz et al., 1993;Ziegler et al., 1993). Mutated p53 is unable to induce apoptosis, therefore allowing for the persistence of DNA damaged cells and mutagenesis.

1.6.2.6.Non melanoma skin cancer

Two mechanisms have been proposed to explain the development of non melanoma skin cancer (NMSC):

- UVR-induced DNA PPs(Brash et al., 1996) and consequent mutation of the p53 gene.

- UVR-induced immunosuppression(Nishigori et al., 1996), which may be mediated by CPDs(Kripke et al., 1992).

The finding of CC to TT tandem mutations in the p53 gene of basal cell carcinomas and squamous cell carcinomas provide evidence of a relationship between UV-induced DNA PPs and NMSC in man(Nakazawa et al., 1994). Xeroderma pigmentosum (XP) patients have an incidence of NMSC which is 1000 fold greater than that seen in normal subjects. XP patients exhibit a deficiency in their ability to repair damaged DNA, this lack of repair of DNA PPs in XP patients further indicates an association between DNA photodamage and human NMSC(Nakazawa et al., 1994;Dumaz et al., 1993;Ziegler et al., 1993).

1.6.3. UVR and NO

1.6.3.1. UVR and NO stores

The first evidence that NO-related products exibit photoproperties was provided by Furchgott *et al.* in 1955, he demonstrated that light had a relaxant effect on vascular smooth muscle(Furchgott et al., 1955).The action spectra for the photorelaxation in rabbit aorta peaked at 310nm with a shoulder at 350nm, following incubation with NO_2^- 355nm dominated the action spectrum(Furchgott et al., 1961). More recently Rodriguez J *et al.,* have further investigated the photochemical properties of NO related substances (table 1.2), using biochemical and optical approaches, they show that the amounts of NO_2^- and RSNOs fully account for the photorelaxation observed in rat aortic tissue. A strikingly close match was observed in the amplitudes of the action spectra for NO release and those of the action spectra for photorelaxation. RSNOs have the highest photoactivity, followed by RNNOs, and NO_2^-; NO_3^- photoactivity is negligible. Consistency between biochemical, photolytical and functional results indicate the presence of two NO stores that dominated the action spectra for photorelaxation: a GSNO-like substance (RSNOs) and NO_2^-. Megson *et al.,* also demonstrate photorelaxation with a GSNO-like substance *in vivo*(Megson et al., 2000). Nitrite probably contributes most highly to enzyme-independent

photorelaxation *in vivo* as it is present in the highest concentration compared with the more photoactive RSNOs(Rodriguez et al., 2003).

Substance	λ max, nm
GSNO	330-340
SNOAlb	310
DMNA	330-370
NO_2^-	310, 350
NO_3^-	302

Table 1.2 *Photochemical properties of NO-related products*(Rodriguez et al., 2003)

The photolytic properties of NO-related products have been utilised to quantify the RSNOs(Rodriguez et al., 2003;Stamler et al., 1992b;Stamler et al., 1992a;Tyurin et al., 2001) and NO_2^- content of media(Rodriguez et al., 2003). Two independent groups have demonstrated a non-linear behaviour of the RSNO/NO pathway, in thiol containing samples photolysis of both NO_3^- and NO_2^- is catalysed by the presence of thiols(Paunel et al., 2005;Dejam et al., 2003). This will undoubtedly have implications for those groups that advocate the use of photolysis in the quantification of NO metabolites in biological samples.

Using *ex vivo* skin biopsy specimens, UVA irradiation has been shown to induce photolysis of RSNO and NO_2^- stored in human skin with subsequent high-output enzyme-independent NO formation. This reaches a maximum 20 minutes after the onset of irradiation(Paunel et al., 2005) (figure 1.13). It has been postulated that this rapid enzyme-independent release of NO post UVR is biologically important and may play a role in regulating UVR-induced keratinocyte apoptosis.

1.6.3.2.UVR and NOS

UVR, predominantly UVB and in some subjects UVA, induces upregulation of iNOS expression in human epidermis. UVR-induced iNOS expression starts 8-10 hours after UVR and is maximal at 24 hours, maximal activity thus corresponds with UVR-induced erythema (figure 1.13). Epidermal iNOS expression returns to baseline three days following UV exposure(Kuhn et al., 1998;Suschek et al., 2001b).

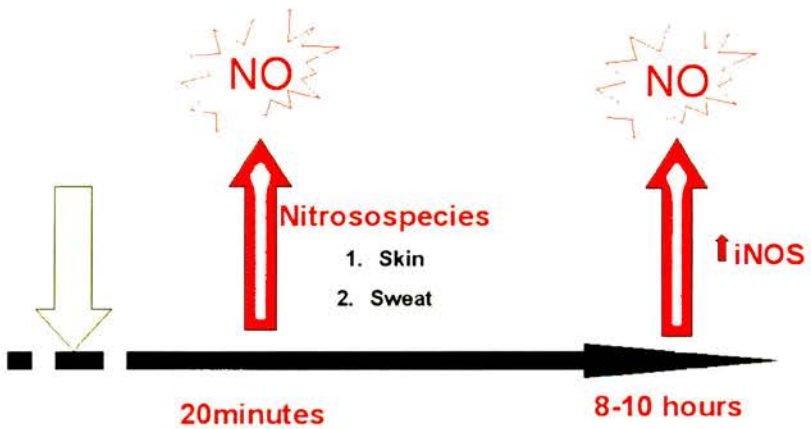


Figure 1.13 *UVR-induced enzyme-independent and dependent NO release*

1.6.3.3.UVR-induced erythema and NO

UVB, the major erythemogenic wavelength of UVR, induces the release of many mediators including NO(Warren, 1994;Goldsmith et al., 1996). It has been shown that UVR-induced erythema is significantly reduced by inhibitors of NOS(Goldsmith et al., 1996). These findings make the quantification of erythema a useful biological measure of NO production.

1.6.3.4.UVR-induced apoptosis and NO

The pleiotropic nature of NO is exemplified when considering its role in cell apoptosis and DNA damage. NO has been shown to exert cytotoxic effects and initiate the onset of apoptosis in mammalian cells(Kroncke et al., 1997), however it has also been shown to protect against the detrimental actions of superoxide,

hydrogen peroxide and alkyl peroxides(Wink et al., 1996b). NO-induced DNA alterations may lead to p53 post-translational modifications, accumulation and activation(Forrester et al., 1996;Messmer and Brune, 1996;Nakaya et al., 2000), thus contributing to the anti-carcinogenic effects of p53. In addition, NO may modulate tumour DNA repair mechanisms by up-regulating poly (ADP-ribose) polymerase (PARP) and the DNA-dependent protein kinase (DNA-PK)(Xu et al., 2002). In contrast, high levels of NO have been shown to mutate the p53 gene allowing for selective clonal expansion of p53-mutant cells, and tumorigenesis(Greenblatt et al., 1994).

Recent evidence suggests that NO has an anti-apoptotic role in skin after exposure to UVR. iNOS derived NO has been shown to protect rat endothelial cells *in vitro* against UVA-induced apoptosis(Suschek et al., 1999). Enzyme independent mechanisms are also important, the presence of NO_2^- *in vitro* has been shown to protect endothelial cells from UVA induced apoptosis, in a concentration-dependent manner. It is postulated that NO_2^- forms NO which acts anti-apoptotically(Suschek et al., 2003b). An anti-apoptotic role for NO has also been demonstrated in human keratinocytes *in vitro* and murine keratinocytes *in vivo* following UVB exposure(Weller et al., 2003). The emerging evidence suggesting an anti-apoptotic role for NO in human keratinocytes following UV exposure, has led to the hypothesis that the rapid enzyme-independent release of NO post UVR is biologically important and potentially 'bridges the gap' between UV challenge and the enzymatic up-regulation and activity of iNOS(Paunel et al., 2005).

1.7. NO donors

The ubiquity of the biological actions of NO offers many promising pharmacologic benefits, however it is at the same time its downfall. The two major practical problems when considering NO as a therapeutic modality are the lack of specificity, which may result in unwanted side effects, and the lack of adequate pharmacokinetically appropriate and localised means of NO delivery.

1.7.1. General NO donor drugs

NO gas is notoriously difficult to handle on account of the problems associated with complete exclusion of oxygen to prevent oxidation to NO_2. NO donors offer the property of stabilising the radical until its release is required. A number of different classes of NO donor drugs already exist, these are categorised by the species derived and the metabolic process underlying NO release(Megson and Webb, 2002).

- The organic nitrates are the most commonly used NO donor drugs in the clinical setting. These include glyceryl trinitrate (GTN) and isosorbide mononitrate (ISMN), they are used for the acute and chronic treatment of angina respectively.
- Sodium nitroprusside (SNP), azide and hydroxylamine require complex metabolism to generate intracellular NO. Release of NO from azide and hydroxylamine is catalase-dependent, while membrane bound proteins are thought to have a role in NO generation from SNP.
- S-nitrosothiols cover a vast array of different compounds which contain a single chemical bond between a SH-group and the NO moiety. Biological activity of RSNOs is highly influenced by the molecular environment of the parent thiol. They can release NO spontaneously, and also have the ability to transfer NO to free reduced thiols and cysteine residues in proteins, thus modulating enzyme activity, this may constitute an important component of their cellular effects(Butler and Rhodes, 1997).
- Diazeniumdiolate, or 'NONOate', compounds are nucleophiles with two molecules of NO, which are spontaneously released in aqueous solution in a temperature and pH-dependent reaction. A wide variety of diazeniumdiolates are available, such as SPER/NO, DEA/NO and diethylenetriamine (DETA/NO) – based compounds, each with a different rate of release of NO that depends on the nature of the nucleophile(Hrabie et al., 1993).
- The syndonimines are frequently used in studies into the effects of NO. These compounds generate equal amounts of NO and O_2^-, which combine rapidly to

form $ONOO^-$; as a result they are generally considered to be $ONOO^-$ donors (Feelisch et al., 1989).

- A novel approach to storage and delivery of NO has recently been adopted using ion-exchanged zeolites (Ze)(Wheatley et al., 2006). These are microporous insoluble materials that form a framework containing metal ions that can bind NO. Zeolite-NO (Ze-NO) produces effects caused directly by NO itself. Ze based compounds are already used clinically as MRI gastrointestinal contrast agents and as clotting enhancers.

1.7.1.1.Cutaneous NO donor drugs

In general the enormous variety of effects of NO in different tissues and systems might be a considerable limitation to systemic NO delivery, with unwanted side effects outside the target tissue. An advantage of studying the effects of NO on human skin is that many of these problems are avoided, as topical application of NO donors should theoretically reduce systemic side effects, while still delivering relevant concentrations of NO to produce a clinical effect. The synthesis of topical NO donors for use on the skin has been a more recent development, these include donors based on:

- The chemical reduction of NO_2^- such as acidified NO_2^-(Ormerod et al., 1999a).
- The spontaneous release by nitroso-sugars(Khan et al., 2003).
- The spontaneous release by diazeniumdiolates(Masters et al., 2002).
- The spontaneous release by RSNOs in a hydrogel(Seabra et al., 2004).
- Release by poly-S-nitrosated polyesters(Seabra et al., 2005).
- Release from iontophoresed iron nitrosyl complexes(Jagren et al., 2002;Webster and Mahajan, 2002).
- Release from a Ze framework of metal ions that can bind NO.

1.7.1.2.Zeolites as NO donor drugs

Zeolites are a class of highly crystalline aluminosilicate microporous materials. They have an inorganic, infinitely extending rigid three-dimensional network of

channels, cages and rings, composed of fully linked, corner-sharing tetrahedra built from an open network composed of AlO_4 and SiO_4. The open structure of the framework allows ion exchange, reversible dehydration and the adsorption of small molecules such as NO. Zeolites are used extensively in washing powder formulations as water softeners, sodium-calcium exchange takes place within the zeolite structure. Zeolites can be used to store gaseous NO, which is released on hydration (as after application to the skin). The kinetics of NO release can be adjusted by varying the structure, metal ion content and vehicle containing the Ze(Wheatley et al., 2006;Xiao et al., 2007). Zeolite-A (LTA) consists of alternating SiO_4 and AlO_4 tetrahedra that share corners to produce an open framework. It has a natural affinity for NO which is bound within the framework, and NO is released (delivered) from this stable storage material in biologically relevant amounts on contact with water. Ze-NO has anti-thrombotic properties, by inhibiting platelet aggregation and adhesion in plasma(Wheatley et al., 2006). The release kinetics of NO from Ze can be tailored by altering the type and number of metal cations in the structure, allowing NO delivery patterns to be modulated to specific clinical requirements. Colleagues in the School of Chemistry, University of St Andrews have recently developed an effective topical NO donor based on Ze.

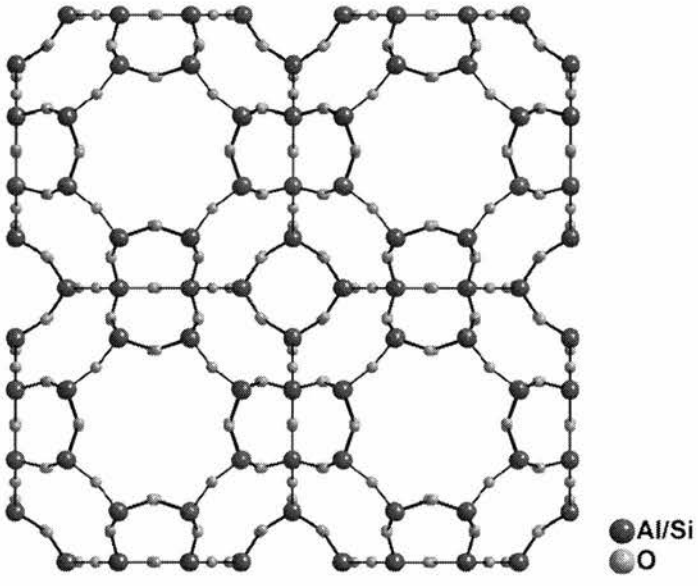


Figure 1.14 *Structure of the zeolite framework*

1.8. Summary

Historically inorganic nitrogen oxides have a long and rich history, however recent advances in the field of NO have only been made since the 1980s. NO plays a major role in human skin, in particular in the context of photobiology. Following UV exposure NO stores present in human skin release NO by enzyme-independent photolysis within 20 minutes of UV exposure. Enzyme-dependent NO release begins 8-10 hours after UV exposure and is maximal by 24 hours. Data from human keratinocytes *in vitro* and murine keratinocytes *in vivo* suggest that NO is having an anti-apoptotic role in human skin following UVR(Weller et al., 2003). NO is biologically active in many systems, however it has pleiotropic actions depending upon its concentration, target organ and microenvironment. The divergent nature of NO make it imperative to direct research towards *in vivo* clinical studies, in order to fully elucidate the role of NO in the normal biological situation.

1.9. Aims

1.9.1. NO donors and inflammation

- To elucidate the inflammatory properties of NO
- To compare the inflammatory effects of NO when delivered by two NO donors:
    - Acidified NO_2^-
    - Ze-NO
- To investigate the suggested inert properties of Ze-NO as an NO donor, and therefore the use of Ze-NO for manipulation of the NO environment in future studies.

1.9.2. NO stores in human skin

- To quantify NO-related products in human skin *in vivo*:
    - In sweat on the skin surface
    - In epidermis
    - In superficial vascular dermis
- To determine *in vivo* the extent to which NO release can be demonstrated during UVA irradiation.

1.9.3. NO and UVR-induced apoptosis, p53 & DNA damage/repair

- To determine the influence of physiologically relevant concentrations of UVR in the presence/absence of exogenous NO on:
    - Apoptosis
    - p53 accumulation
    - DNA damage and repair

1.9.4. NO, UVB and arginase in human skin

- To further investigate preliminary findings which suggest a feedback control mechanism between iNOS and arginase, competing for the common substrate L-Arginine.

CHAPTER 2
METHODS

2. CHAPTER 2 - METHODS

This chapter includes all methods which are common to more than one experiment. All other methods are included within the methods section of the individual chapters.

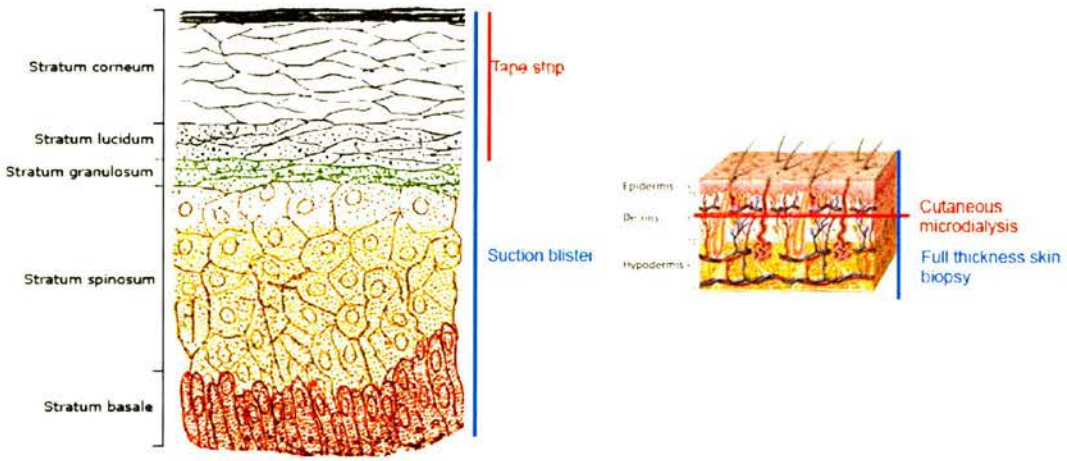
2.1. Study volunteers

All the studies used healthy volunteer subjects. All subjects had Fitzpatrick skin type I or II, except for the *NO/UVB-induced upregulation of arginase study* in which one subject had skin type IV. None of the subjects were using either topical or systemic medications at the time of the study. The Lothian Regional Ethics Committee approved all the studies and all volunteers gave written informed consent. All the studies were conducted according to the principles of the declaration of Helsinki.

2.2. Preparation and application of topical NO donors

Zeolite manganese NO (Ze-NO) was used as a NO donor in all studies. This is an inert topical formulation which releases NO on mixing Ze-NO powder with an aqueous vehicle(Mowbray et al., 2008). Immediately before application Ze-NO powder was mixed with aqueous cream BP® and applied to the skin surface. Aqueous cream BP® was used as a vehicle control in all experiments. All topical preparations were occluded with Tegaderm™ after each application. Ze-NO donor was mixed at a concentration of 33% wt/wt for all experiments. A 33% wt/wt formulation comprises the maximum ratio of Ze-NO powder to aqueous cream, while maintaining the ability to apply the formulation to the skin surface.

2.3. Methods of sampling of human skin



a) Epidermis ***b) Full thickness epidermis and dermis***

Figure 2.1 ***a & b Sites of human skin sampling using different methods***

2.3.1. Epidermal tape stripping

D-Squame® (CuDerm Corp., Dallas, TX, USA) strips, diameter 22mm, surface area 3.8cm^2, were used to sample the stratum corneum (superficial epidermis, figure 2.1a). Strips were placed on the skin and the skin marked to ensure sampling from the same site. Equal pressure was applied to each strip by pressing with a spring loaded stamp for two seconds duration, strips were then removed with tweezers and stored on a cellophane sheet at -70^{0}C until further analysis (figure 2.2).

Figure 2.2 *Epidermal tape strip materials and method*

2.3.2. Epidermal suction blister and fluid, harvest and homogensation

Up to ten epidermal suction blisters were formed on the volar forearm of each volunteer using two suction blister cups (dermovac®, Ventipress Oy, Lappeenranta, Finland). Each cup creates five 5mm diameter blisters, the split occurs at the dermo-epidermal junction (figure 2.1a & b, figure 2.3). Epidermal suction blister fluid was removed using a sterile 1ml insulin syringe (BD PlastipakTM), the blister cavity was then filled with Xylocaine® 1% with adrenaline (epinephrine) 1:200.000 (Astra Zeneca LTD.,Luton, UK). The blister roof was harvested aseptically using fine scissors. *Epidermal suction blister samples* were placed immediately into ceramic bead beating tubes (CK-14, Stretton scientific Ltd., Stretton, UK) containing NEM/EDTA/trypsin. *Epidermal suction blister fluid* was placed immediately into eppendorfs containing NEM/EDTA. A Precellys 24 homogeniser (SS-Bertin-01, Stretton scientific Ltd., Stretton, UK) was used to homogenise the epidermal suction blister samples, two cycles with a three minute break (on ice) at 5,500 revolutions/minute (a cycle consists of three 20 second active periods with 30seconds rest between each). Post-homogenisation, samples were centrifuged and supernatant transferred into eppendorfs for storage prior to quantification of NO-related products.

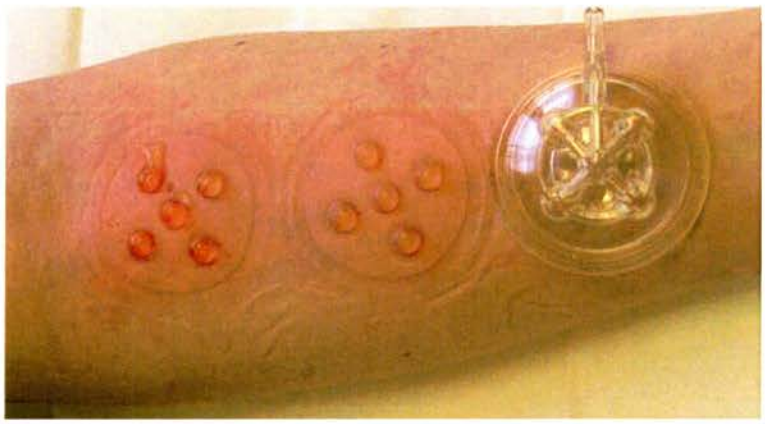


Figure 2.3 *Epidermal suction blister and blister cup*

2.3.3. Full thickness punch biopsy

In all experiments where full thickness skin biopsies were taken a 4mm diameter sterile disposable punch biopsy was used (Stiefel® Laboratories, Bucks, UK). Skin was anaethetised with an intradermal injection of 1% lignocaine with adrenaline prior to biopsies being taken. Biopsy wounds were closed using 4/0 ethilon sutures (Ethicon, Johnson and Johnson medical Ltd., W Lothian, UK). All 4mm full thickness skin punch biopsies were fixed in 4% buffered formaldehyde prior to processing for histology.

2.3.4. Cutaneous microdialysis sampling of superficial vascular dermis

Dermal microdialysis catheters were made by the technique of Clough(Clough et al., 1998) and sterilized by ethylene-oxidisation. Sites on the volar aspect of the forearm and the flexor aspect of the upper arm were used for catheter insertion. Prior to catheter insertion sites were anaesthetized with topical 5% EMLA® cream, applied for 1 hour. Using the Seldinger technique and a 23-gauge needle (BD Microlance™ 0.6mm x 2 5mm, Consumer Healthcare, Franklin Lakes, New Jersey), microdialysis catheters were inserted into the superficial dermis parallel with the skin surface (figure 2.1b & 2.4). Either normal saline or Noradrenaline (1:1,000 Abbott laboratories Ltd Kent, UK) was dialysed continuously through the catheters at a rate of 2μL/min, using 1ml Micro-Fine™ insulin syringes (BD Consumer Healthcare, Franklin Lakes, New Jersey) and a microinfusion pump (PHD 2000 infusion pump, Harvard Apparatus, Holliston, M.A.). Noradrenaline was diluted 1:80 with normal saline. Dialysate was collected continuously in 15 minute aliquots throughout each experiment. Following insertion of the microdialysis catheters dialysate was collected for 30 minutes prior to manipulation of the environment (eg UVR, NO donor application).

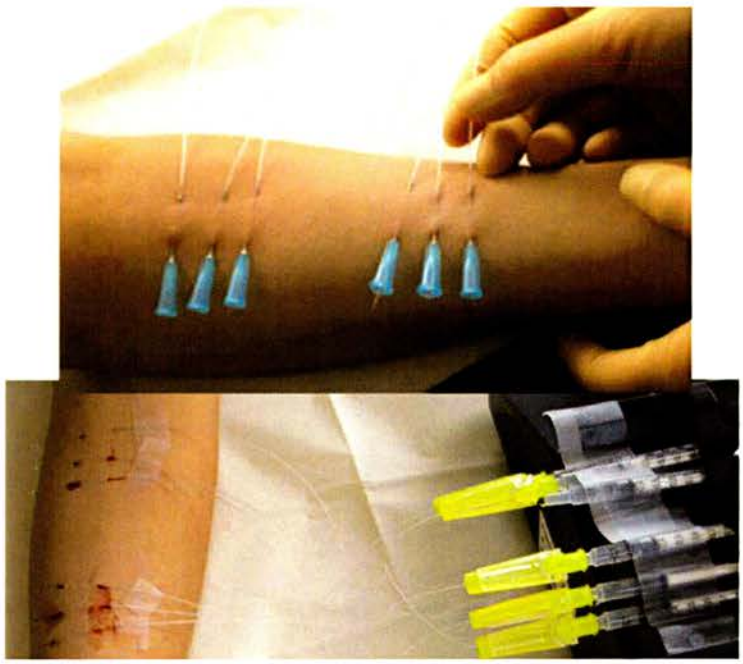


Figure 2.4 *Cutaneous microdialysis, a method of sampling from superficial vascular dermis*

2.4. Determination of the protein concentration of biological samples

When necessary protein concentrations of biological samples in solution were determined using a colourimetric Bradford protein assay (Bradford reagent, Bio-Rad Labs., Hercules, CA) in a microtiter plate format (Tekin S, Dept Animal Sciences, University of florida). Samples were analysed in triplicate at dilutions of: 1:60, 1:80 and 1:100.

2.5. Chemiluminescence Assay for nitrate, nitrite and NO-related products in biological samples

Total NO-related products and individual concentrations of NO_3^-, NO_2^-, RSNOs and RNNOs were quantified in solutions of various biological samples. All samples were frozen at -70^{0}C immediately after collection, and analysed within three days. Cutaneous microdialysis samples were analysed within 24 hours of collection for total NO-related products only, due to the small sample volume. Unless otherwise indicated samples were kept on ice in the dark during processing. Epidermal suction blisters were stored in N-ethylmaleimide (NEM)/ethylenediamine tetra acetic acid

(EDTA)/trypsin prior to analysis (final concentration 5mM/2mM/x1). Suction blister fluid, sweat, blood and saliva samples were stored in NEM/EDTA (final concentration 5mM/2mM). NEM alkylates free thiols and stabilises RSNOs preventing any further S-nitrosation(Marley et al., 2000). EDTA forms inert complexes with transition metals, several of which can accelerate RSNO degradation even at low concentration(Mcaninly et al., 1993).

NO_3^-, NO_2^-, RSNO and RNNO concentrations were determined by reductive denitrosation with an iodide/triiodide containing reducing mixture and subsequent measurement of the gaseous NO released by its chemiluminescent reaction with O_3, as described by Feelisch *et al.*(Feelisch et al., 2002;Marley et al., 2000) (figure 1.7). Samples (volume 10-100µl) were introduced using a gastight syringe (SGE International Pty Ltd, Ringwood, Australia) into a sealed reaction vessel containing the reducing mixture of 45mmol/l potassium iodide (KI) and 10mmol/l iodine (I_2) in glacial acetic acid. A constant flow of nitrogen gas was bubbled through the reaction vessel which was maintained at a temperature of 60^0C in a thermostatically controlled water bath (Grant Instruments (Cambridge) Ltd, England). The effluent gas was passed through a scrubbing bottle containing sodium hydroxide (1 mol/L; 0^0C) to trap traces of acid and iodine before transfer into the chemiluminescence meter (42C NO-NO_2-NO_X Analyser, Thermo Environmental Instruments Inc, Franklin, Massachusetts). The chemiluminescence meter was connected to an analogue-digital signal converter (PowerLab® 2/25 ADInstruments, Castle Hill, NSW, Australia) and the output processed by PowerLab® System Chart & Scope for Windows® (v5.1.1, AD Instruments).

Without having to change reduction mixture or conditions, NO_3^-, NO_2^-, RSNOs and RNNOs can be differentiated by pre-treatment with group specific reagents before analysis according to the technique of Feelisch *et al.*(Feelisch et al., 2002) (chapter 1.5.3.1, figure 1.8). All individual NO-related products were quantified in terms of nitrite equivalents so as to allow for direct comparison of each individual product.

2.5.1. Nitrite Assay

NO_2^- measurements were made by dividing samples into two aliquots. Direct injection of one aliquot into the tri-iodide reducing mixture measured NO derived from NO_2^-, RSNO and RNNO. Sulfanilamide reacts specifically with nitrite to form a stable diazonium ion which is unaffected by the reducing mixture. The second aliquot was pretreated with 10% (v/v) of a 5% solution of sulphanilamide in 1N HCl (final concentration 29mmol/L) and incubated for 15 minutes at room temperature (RT). Subtracting pre-treated aliquot derived NO from the untreated aliquot derived NO gave the amount of NO_2^-.

2.5.2. RSNO assay

Incubation with $HgCl_2$ results in cleavage of the S-NO bond (Saville reaction) (Feelisch et al., 2002) without affecting peak shape or recovery of NO_2^- or NO. RSNO concentration in each sample was quantified by subtraction of the peak area of sample aliquots pretreated with a solution of sulfanilamide and mercuric chloride at RT for 30 minutes (10% (v/v) of 5% sulphanilamide + 0.2% $HgCl_2$ in 1N HCl (final concentration 7.3mmol/L)) from that of sample aliquots just treated with sulphanilamide at RT for 15 minutes (10% (v/v) of 5% sulphanilamide in 1N HCl).

2.5.3. RNNO or metal nitrosyls assay

The peak remaining after preincubation with $HgCl_2$/sulphanilamide/H^+ represents the presence of RNNOs or metal nitrosyls in the sample.

2.5.4. Nitrate assay

NO_3^- was reduced to NO_2^- by enzymatic reduction(Schmidt.H and Kelm.M, 1996). A 1:30 mix of NO_3^- reductase solution:biological sample was incubated at 37^0C for 15 minutes. The NO_3^- reductase solution was premixed using *Aspergillus* purified NO_3^- reductase (final concentration 0.1U/ml) (SIGMA-ALDRICH, Germany), flavine adenine dinucleotide (FAD, final concentration 5µM) (SIGMA-ALDRICH,

Germany) and reduced β-nicotinamide dinucleotide phosphate (NADPH, final concentration 0.03mM) (SIGMA-ALDRICH, Germany) in double distilled water. The amount of NO_3^- was quantified by subtracting the peak area of the untreated aliquot from that of the aliquot pre-treated with NO_3^- reductase solution.

Prior to the analysis of each batch of biological samples, a range of NO_2^- standards (Sodium NO_2^- , AnalR, BDH Chemicals Ltd, UK) were injected into the reaction vessel in order to calibrate the gaseous NO released as equivalent NO_2^- concentration. Assay reproducibility was determined by comparison of the peak area from each of the NO_2^- standards ($1x10^{-3}$ – $1x10^{-8}$mMoles) measured in triplicate (figure 2.5).

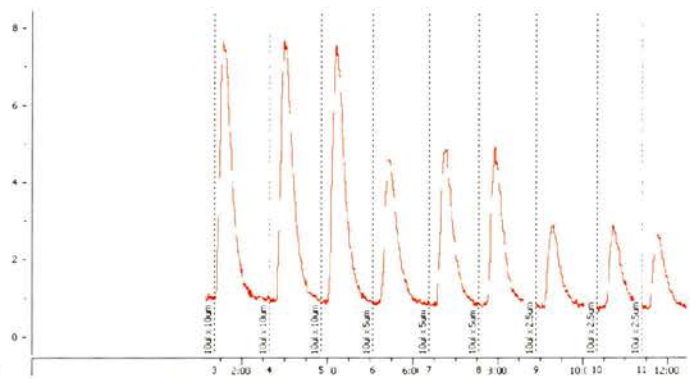


Figure 2.5 *Powerlab tracing of chemiluminescent analysis of nitrite standards analysed in triplicate*

Nitrite standards were analysed at the beginning and end of each set of biological samples to ensure reproducibility throughout the period of analysis (figure 2.6).

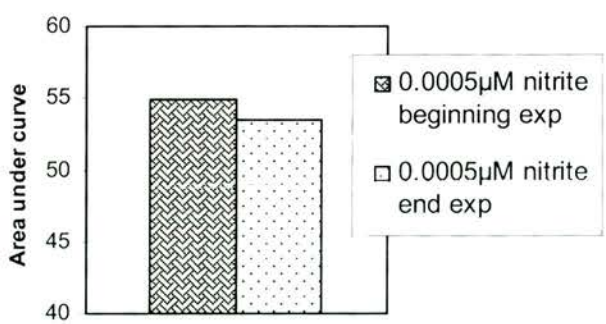


Figure 2.6 *Reproducibility of nitrite standards analysed before and after a set of biological solutions*

Analysis revealed a small amount of degradation in NO_2^- standards over time, therefore NO_2^- standards were replaced every two weeks and new standards compared with the old before discarding the latter.

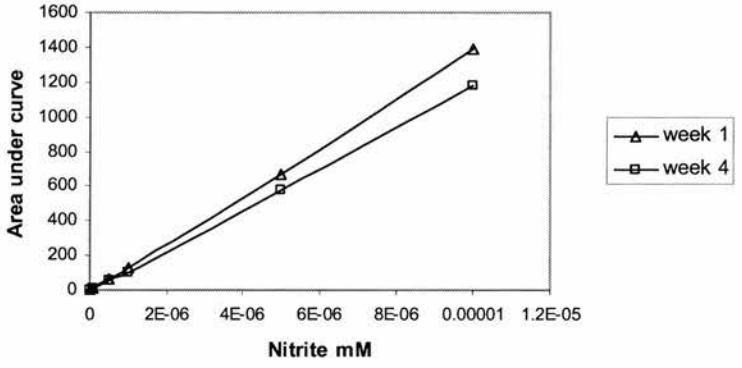


Figure 2.7 *Degredation of nitrite standards over a four week period*

All gastight syringes were washed out with acetone between sample batch analysis to minimise NO_2^- and organic contaminants. Control solutions of NEM/EDTA were analysed for contamination with NO-related products where appropriate.

Many laboratory materials have the potential for contamination with minute quantities of NO_2^- which may affect results. All vehicle solutions were analysed to ensure that there was no NO_2^- contamination which would influence the results. It is not possible to ensure zero measurements of NO_2^- within such laboratory solutions, as the method of gas-phase chemiluminescence is so sensitive. Minimal contamination of laboratory solutions was observed, as can be seen by comparison with resting NO_2^- concentrations in the superficial vascular dermis of unirradiated skin (figure 2.8).

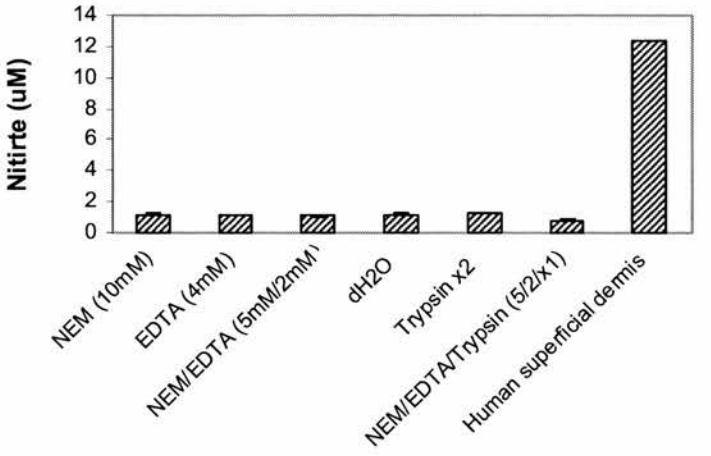


Figure 2.8 *Nitrite concentration measured in laboratory/vehicle solutions*

2.6. Determination of minimal erythemal dose (MED)

For all studies requiring assessment of the MED prior to commencement of the study, subjects attended on day one where they received five graded doses of UVB to five individual areas on the volar aspect of the dominant forearm, each measuring 1 x 2cm^2. The MED range used was matched to body site and skin type using a departmental protocol(Waterston et al., 2004). Subjects returned 24 hours later to have their MED read, a subjects MED was defined as the minimal dose producing uniform erythema with clearly defined template margins.

2.7. Statistical analysis

Data for individual subjects are presented as means of duplicate or triplicate samples. Pooled data is presented as means ± standard error of the mean (SEM) and differences judged as significant if the p-value was lower than 0.05, as determined by the paired two tailed Student's t-test. Analysis of variance (ANOVA) was used to determine significant differences between three or more observations. ANOVA tests the hypothesis that means from two or more samples are equal. Box plots showing the median and inter-quartile range (IQR) with points representing maximum,

minimum and mean values are used to display inter individual variation. Relationships between an independent variable (eg plasma NO-related products) and a dependent variable (eg sweat or superficial dermal NO-related products) are shown by regression analysis. The proportion of variation is displayed by the (correlation coefficient)^2 (R^2). Data analyses were conducted using Microsoft® Excel Software, v5 and MINITAB® release 14 for windows.

2.8. Selecting a method of tissue homogenisation

All biological samples to be analysed for gas-phase chemiluminescence quantification of individual NO-related products are required to be in the form of a solution. Various methods of homogenisation were trialed prior to selecting the most effective method. Both full thickness skin biopsies and in particular epidermal suction blisters pose a challenge for homogenisation. The main problem in achieving adequate homogenisation lies in the elastic nature of the tissue. Methods which were compared for their effectiveness at fully homogenising tissue include (with and without prior trypsinisation):

- Sonicator
- Tissue tearor (mini liquidizer)
- Precellys 24 (glass bead whirlimixer)

A number of factors are important to bear in mind when selecting the most appropriate method of homogenisation. All samples are being prepared for quantification of individual NO-related products. In order to ensure that results accurately demonstrate the *in vivo* distribution of NO-related products, samples should be kept in the dark and below 4^0C at all times, this ensures maximum stability of the individual NO-related products and an accurate representation of their *in vivo* state. More obvious requirements include selection of the method that provides the most uniform and reproducible homogenisation with minimal loss of biological sample.

2.8.1. Homogenisation using a sonicator

This was the first method of homogenisation to be trialed. Sonicator homogenisation utilizes focused high frequency acoustic waves to create a powerful vortex. This process was performed in a dark room, samples were stored on ice between cycles. Unfortunately this did not prove to be a useful method for a number of reasons:

- Samples required multiple cycles of treatment to achieve maximal homogenisation (3 x 3 second pulses repeated 6 times → 30 minute rest on ice → 3 x 3 second pulses repeated 6 times). Each cycle of treatment resulted in heating of the biological sample, despite introducing a long rest period on ice. Such heating will potentially destabilise individual NO-related products.
- This method results in frothing of the biological sample which led to occasional loss of tissue.
- Despite maximizing the effectiveness of the homogenisation process as best as possible, there remained high variability in effectiveness of homogenisation between samples. This variability could be observed visually simply by inspecting the sizes of tissue specimens suspended within the sample solutions.

2.8.2. Homogenisation using a tissue tearor

The tissue tearor (Biospec products,Inc., Oklahoma) is a rotor/stator type tissue homogeniser which rapidly homogenises, disperses, and emulsifies samples in 0.5 - 50 ml of liquid. The rotor turns at 5-30,000 rpm. Although not ideal, use of the tissue tearor with prior trypsinisation of samples for 1 hour proved more reliable than homogenisation using the sonicator. Some problems still remain with the use of the tissue tearor for homogenisation, these include:

- Occasional loss of tissue from the eppendorf.
- Tissue can get stuck between the rotating blade and the protective outer casing without being homogenised.
- This method requires repetitive cycles at the highest speed, on ice, resulting in a duration of processing for 10 suction blister samples of 2 hours. The time

taken to process individual samples increases the potential of exposure to variations in temperature/light which may destabilise NO-related products.

- Variability in the effectiveness of homogenisation was again observed, some samples of tissue remained visibly larger than others.

2.8.3. Homogenisation using the Precellys 24

Precellys 24(SS-Bertin-01, Stretton scientific Ltd., Stretton, UK). Biological samples are introduced into sterile ceramic bead beating tubes. High speed whirlimixing in a 'figure of eight motion' results in the rapid, uniform homogenisation of skin tissue samples. The final biological solution is homogenous and milky-white, no residual tissue fragments are visible. Twenty-four samples can be processed at any one time. The effective homogenisation of skin tissue requires two cycles with a 3 minute break between each (on ice) at 5,500 revolutions/minute (a cycle consists of three 20 second active periods with 30 seconds rest between each). Homogenisation using the Precellys 24 tissue homogenizer was chosen as the desired method of tissue homogenisation for a number of reasons:

- Rapid processing of a number of samples at one time, thus minimising exposure to both heat and light.
- No loss of biological tissue.
- Less variability of homogenisation between samples in comparison with other methods trialed, demonstrated both by visual inspection and by variation in protein concentration of samples (figure 2.9).

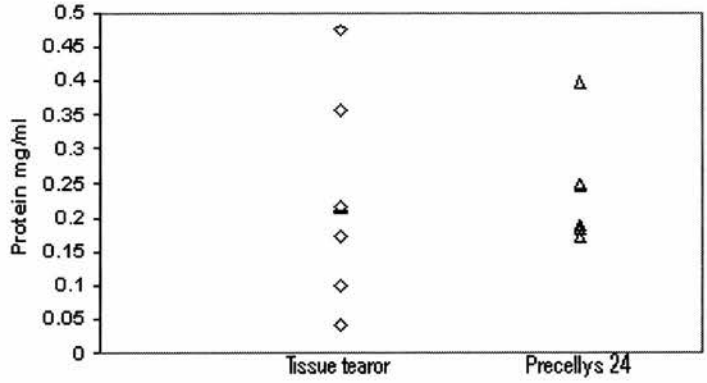


Figure 2.9 *Comparison of homogenisation using Tissue tearor and Precellys 24*

Tissue tearor mean 0.21±0.16mg/ml protein, SEM 0.06

Precellys 24 mean 0.24±0.05mg/ml protein, SEM 0.02

CHAPTER 3
NO DONORS
AND
INFLAMMATION

3. CHAPTER 3 - NO DONORS AND INFLAMMATION

3.1. Introduction

NO is well known for its divergent effects, in particular in the processes of apoptosis and inflammation. The effects of NO are dependent on a number of factors, including: the source of NO, the redox form and pH of the microenvironment, and the target cells in tissue. The pleiotropic actions of NO are exemplified when studying the role it plays in inflammation.

NO has many anti-inflammatory properties:

- NO diminishes endothelial permeability(Granger and Kubes, 1996).
- NO donors reduce oedema formation in various experimental models(Hinder et al., 1999).
- NOS inhibitors can exacerbate oedema formation(Mundy and Dorrington, 2000).
- Leukocyte and platelet adherence to the endothelium are inhibited by NO(Clancy et al., 1992;Kubes et al., 1991).
- The production of superoxide anions by leukocytes is inhibited by NO(Clancy et al., 1992).
- NO inhibits macrophage degranulation(Clancy and Abramson, 2000).

Evidence also exists of pro-inflammatory properties of NO:

- In experimental models of inflammation NO has been shown to promote carrageenin-induced oedema in the mouse footpad(Ianaro et al., 1994).
- NO induces vasodilation in the neurogenic inflammatory reaction of the rat hindpaw skin to topical application of mustard oil(Lippe et al., 1993).
- Inducible NOS protein has been demonstrated immunohistochemically in many inflammatory diseases including a number of different inflammatory dermatoses: psoriasis(Kolb-Bachofen et al., 1994;Sakai et al., 1996), atopic dermatitis(Clancy et al., 1998;Rowe et al., 1997) and LE(Clancy et al., 1998).

Subsequent to the isolation of iNOS in psoriasis, contact dermatitis and atopic dermatitis, Ormerod *et al.* studied the effects of topical acidified NO_2^- on the skin(Ormerod et al., 1999a). This NO donor was made by mixing ascorbate and NO_2^- at concentrations of either 0.5% or 5% with a vehicle control of aqueous cream(Ormerod et al., 1999a). An intense inflammatory infiltrate with CD3, CD4, CD8, CD68 and neutrophil elastase expressing cells was seen at sites of acidified NO_2^- application, this set the paradigm that NO is a pro-inflammatory mediator in human skin.

Synthesis of topical NO donors for use on the skin is a recent development. An important feature of such donors is the inert nature of the carrier molecule or vehicle. Zeolites are a class of highly crystalline aluminosilicate microporous materials. They have an inorganic, infinitely extending rigid three-dimensional network of channels, cages and rings, composed of fully linked, corner-sharing tetrahedra built from an open network composed of AlO_4 and SiO_4. The open structure of the framework allows ion exchange, reversible dehydration and the adsorption of small molecules such as NO. Zeolites are used extensively in washing powder formulations as water softeners and are generally regarded as being inert. We have used Zeolites to store gaseous NO, which is released on hydration (as after application to the skin). The kinetics of NO release can be adjusted by varying the structure, metal ion content and vehicle containing the Ze(Wheatley et al., 2006;Xiao et al., 2007). Dr Weller's research group has experience with the topical NO donors acidified NO_2^- (Weller et al., 1998), RSNOs(Seabra et al., 2004) and Ze-NO, and have observed little clinical inflammation following RSNO and Ze-NO application. This suggests that NO itself is not as potent a pro-inflammatory mediator as the early data shows, but that the release of species other than NO might have accounted for the inflammatory effects of acidified NO_2^-.

Since the first description of enzyme-independent production of NO on the skin surface of man a decade ago(Weller et al., 1996), the range of functions in skin in which the involvement of NO has been described has grown. Topical NO donors

have been shown to be mediators in the treatment of cutaneous fungal and viral disease(Ormerod et al., 1999a;Weller et al., 1998), Raynaud's phenomenon(Tucker et al., 1999), diabetic wound healing(Witte et al., 2002b) melanogenesis(Romero-Graillet et al., 1996;Romero-Graillet et al., 1997) and the control of keratinocyte apoptosis following UVR(Weller et al., 2003;Suschek et al., 1999;Suschek et al., 2001a). However, the development of topical NO donors for therapeutic uses such as the encouragement of wound healing, or as anti-apoptotic agents, has been discouraged by the early suggestion that it might be a potent inducer of inflammation. The advent of Ze-NO, a donor of pure gaseous NO, now allows me to revisit the question of NO's effects on healthy human skin.

3.2. Aims

The aim of this study was:

- To elucidate the inflammatory properties of NO
- To compare the inflammatory effects of NO when delivered by two NO donors:
    - Acidified NO_2^-
    - Ze-NO
- To investigate the observed inert properties of Ze-NO as an NO donor, and therefore the use of Ze-NO for manipulation of the NO environment in future studies.

3.3. Methods

All methods common to more than one study are detailed in Chapter 2.

3.3.1. Study volunteers

Four individual studies were performed, each using healthy volunteers:

- Determination of biologically equivalent doses of Ze-NO and acidified NO_2^- - three subjects.
- Assessment of cutaneous inflammation following application of topical NO donors - six subjects (three male).
- Determination of NO delivery to the superficial dermis by NO donors - six subjects (three male).
- Measurement of IL-4 in epidermal suction blister fluid - three subjects. Four subjects participated in the study measuring IFN γ levels (three applied Ze-NO with two of the three and one additional volunteer repeating the procedure to attain control samples).

3.3.2. Determination of biologically equivalent effects of Ze-NO and acidified NO_2^-

It has previously been shown by our group that dermal blood flow correlates directly with the concentration of NO delivered transepidermally from topically applied RSNO NO donors(Seabra et al., 2004). Using this technique the concentrations of acidified NO_2^- and Ze-NO which would deliver the same amount of NO to human skin were determined. Biologically equivalent concentrations of acidified NO_2^-, and Ze-NO, were determined by assessing the erythema induced by application of varying volumes, and concentrations, of each product. Both products were reconstituted in aqueous cream BP®: acidified NO_2^- 0.5%, acidified NO_2^- 5% (each with 2% ascorbic acid), and Ze-NO 20%, 30%, 40% and 50%. Either 0.02ml or 0.04ml of the topical donors were applied to a $1cm^2$ area on the volar forearm, the area was covered with tegaderm™ (3M Health Care, St Paul). Laser doppler probes were secured to the tegaderm overlying each application. Cutaneous blood flow, measured as red blood cell flux using a laser doppler perfusion monitor (Moor Instruments Ltd, Axminster, UK), was used as an index of erythema. The laser doppler unit consisted of one main unit and two satellite units connected to the

server, thus allowing flux readings from three laser probes to be recorded simultaneously. The perfusion monitor was connected to a PC and recordings displayed continuously by MoorSoft for windows/moorLAB v1.31 (Moorsoft Instruments Ltd). At three minute intervals for 120 minutes, real-time recording was paused and the mean blood flow was recorded, a mean of three ten second duration readings was taken at each three minute interval.

3.3.3. Preparation and application of topical NO donors

To assess cutaneous inflammation following topical NO donor application the following formulations were prepared using aqueous cream BP® as a vehicle:

1. 0.02ml Zeolite zinc (33% wt/wt)
2. 0.02ml Zeolite manganese NO (33% wt/wt)
3. 0.04ml Acidified NO_2^-, ascorbic acid (2% wt/wt) + NO_2^- (5% wt/wt)
4. 0.02ml Ascorbic acid (2% wt/wt)

NO_2^- was not tested alone. Each preparation was applied to a $1cm^2$ area of skin on the volar aspect of the forearm eight hourly for two consecutive days. The last application of cream was made 12 hours before assessment of the response and biopsy.

3.3.4. Tissue specimens

Forty-eight hours after application of the NO donors and control 4mm full thickness skin punch biopsies were taken from the centre of each site of topical application. Tissues were embedded in paraffin and 4μm sections were cut. Sections were stained with haematoxylin-eosin (H&E) or processed for immunohistochemical staining using a Dako EnVision™ K5007 system (DakoCytomation Carpintera, California) with subsequent 3,3'diaminobenzidine (DAB) incubation. The EnVision™ system is a two-step procedure: the primary antibody incubation is followed by incubation with an enzyme and antibody labelled inert 'spine' molecule of dextran.

3.3.5. Immunohistochemical quantification of inflammation in epidermis and dermis post topical NO donor application

A panel of antibodies were used for immunohistochemical stainings: CD1a - Dako M3571, CD3 - Dako A0452, CD4 - Novocastra NCL-L-CD4-368 (Novocastra,UK), CD8 - Dako M7103, CD68 - Dako M0876, myeloperoxidase - Dako A398 (for neutrophils and other myeloid cells). Following incubations with the primary antibody and Envision the peroxidase label is visualised with Dako DAB solution with hydrogen peroxide substrate at pH 7.4. Finally slides were counterstained in Haematoxylin before coverslipping. For analysis of the staining results and preparation of images a multi-colour microscope (Leitz), Q imaging monochrome camera and Q Capture Pro computer software (Media Cybernetics, UK) were used. Positive and negative cells were counted by a blinded observer at high magnification using a x40 objective lens.

3.3.6. Quantification of NO delivery to superficial dermis using cutaneous microdialysis

In six subjects, dermal microdialysis catheters were used to measure NO concentrations within the superficial dermis. Six microdialysis catheters were inserted into the superficial dermis of the anaesthetised sites (two at each site). Noradrenaline was dialysed through each catheter at a rate of 2μL/min. The infusion of noradrenaline was maintained throughout the experiment in order to prevent the removal of NO by the cutaneous microvasculature. Dialysate was collected continuously in aliquots covering 15 minute periods. Samples were immediately stored at -70^{0}C for future analysis. Following insertion of the microdialysis catheters dialysate was collected for 30 minutes prior to the application of: 0.02ml Ze-NO (catheters 1 and 2), 0.04ml acidified NO_2^- (catheters 3 and 4) and 0.02ml aqueous cream (catheters 5 and 6), thereafter sampling was continued for a further 90 minutes in 15 minute aliquots.

3.3.7. Determination of NO_2^- concentration in dialysate

All dialysate samples were analysed within 24 hours of collection. Nitrite concentrations were determined by reductive denitrosation by an iodide/triiodide containing reducing mixture and subsequent measurement of the gaseous NO released by its chemiluminescent reaction with O_3 (Ch2.5).

3.3.8. Identification of CD4+ T_H cell-type in epidermal suction blister fluid

For analysis of IL-4 and IFN γ in suction blister fluid volunteers applied 33% Ze-NO and aqueous cream to two separate $25cm^2$ areas on the volar aspect of the forearm. Applications were repeated eight hourly for 48 hours after which topical applications were removed and skin was cleaned with an alcohol wipe (Alcotip swab, Universal Hospital supplies Ltd, UK). Five 5mm diameter epidermal suction blisters were created overlying each of the two sites of topical application. IL-4 and IFN γ concentrations in epidermal blister fluid were analysed using Human IL-4 and Endogen Human IFN γ ELISA kits (Pierce Biotechnology, Inc., Rockford, IL). Protein concentrations of epidermal suction blister fluid were determined. All results were expressed as cytokine concentration (pg) per mg protein.

3.4. Results

3.4.1. Topically applied Ze-NO (0.02ml of 33%) and acidified NO_2^- (0.04ml of 5%) produce similar increases in dermal blood flow and NO

Our group have previously shown that dermal blood flow correlates directly with the concentration of NO delivered trans-epidermally(Seabra et al., 2004). A range of different concentrations of acidified NO_2^- and Ze-NO were applied to a $1cm^2$ area on the volar forearm, subsequently blood flow was measured at each site. Based on this bioassay, 33% Ze-NO was found to produce comparable effects on dermal blood flow as 5% acidified NO_2^- (figure 3.1).

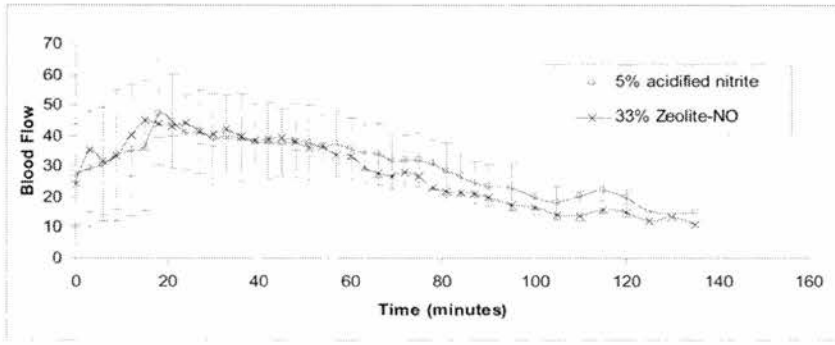


Figure 3.1 Erythema measured with a laser Doppler flow meter following application of topical 33% Ze-NO and 5% acidified NO_2 (n=6, ± SEM)

Microdialysis was then used to measure transepidermal NO diffusion by these two donors (figure 3.2). Dialysate was collected continuously for 60 minutes in four equal aliquots of 15 minutes each. Nitric oxide is oxidised to NO_2^- in aqueous solution, the dialysate NO_2^- was reduced back to NO by tri-iodide reduction and 'stripped' in nitrogen carrier gas to the chemiluminescence analyser for analysis. As with erythema development, comparable release of NO was observed between the two different NO donors, although slightly higher concentrations of NO were recorded from the Ze-NO treated site, this was not significant.

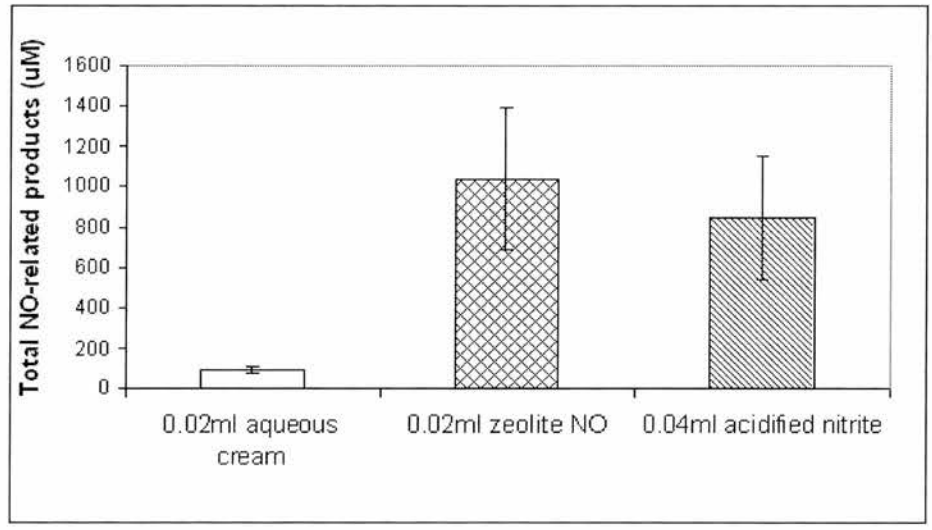


Figure 3.2*NO delivered to superficial dermis by topical 33% Ze-NO and 5% acidified NO_2^-, measurements made by chemiluminescence analysis of cutaneous microdialysate fluid (n=6, ± SEM)*

3.4.2. Acidified NO_2^- causes marked erythema, oedema and ulceration in comparison with Ze-NO and controls

In all six subjects clinical inflammation was observed at the sites of acidified NO_2^- application with significant discomfort, erythema, oedema and superficial cutaneous ulceration. This developed in the first 36 hours of application. A representative example is shown in figure 3.3. The Ze-NO used contains the metal ion manganese (Mn) which induced orange discolouration, attributable to the delocalized electrons within the metal-ligand Mn-NO bond, but there were no clinically visible changes or discomfort consistent with inflammation.

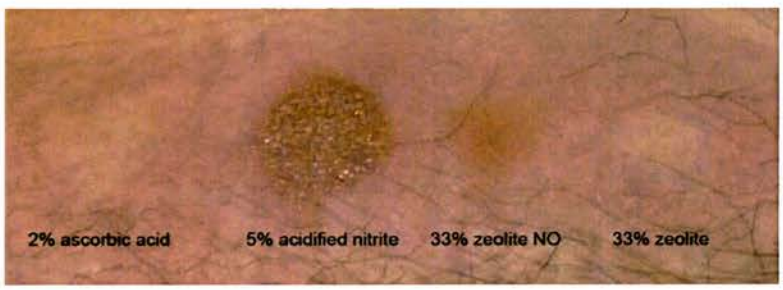


Figure 3.3 *Clinically visible cutaneous inflammation following topical acidified NO_2^-, but not Ze-NO application (n=6)*

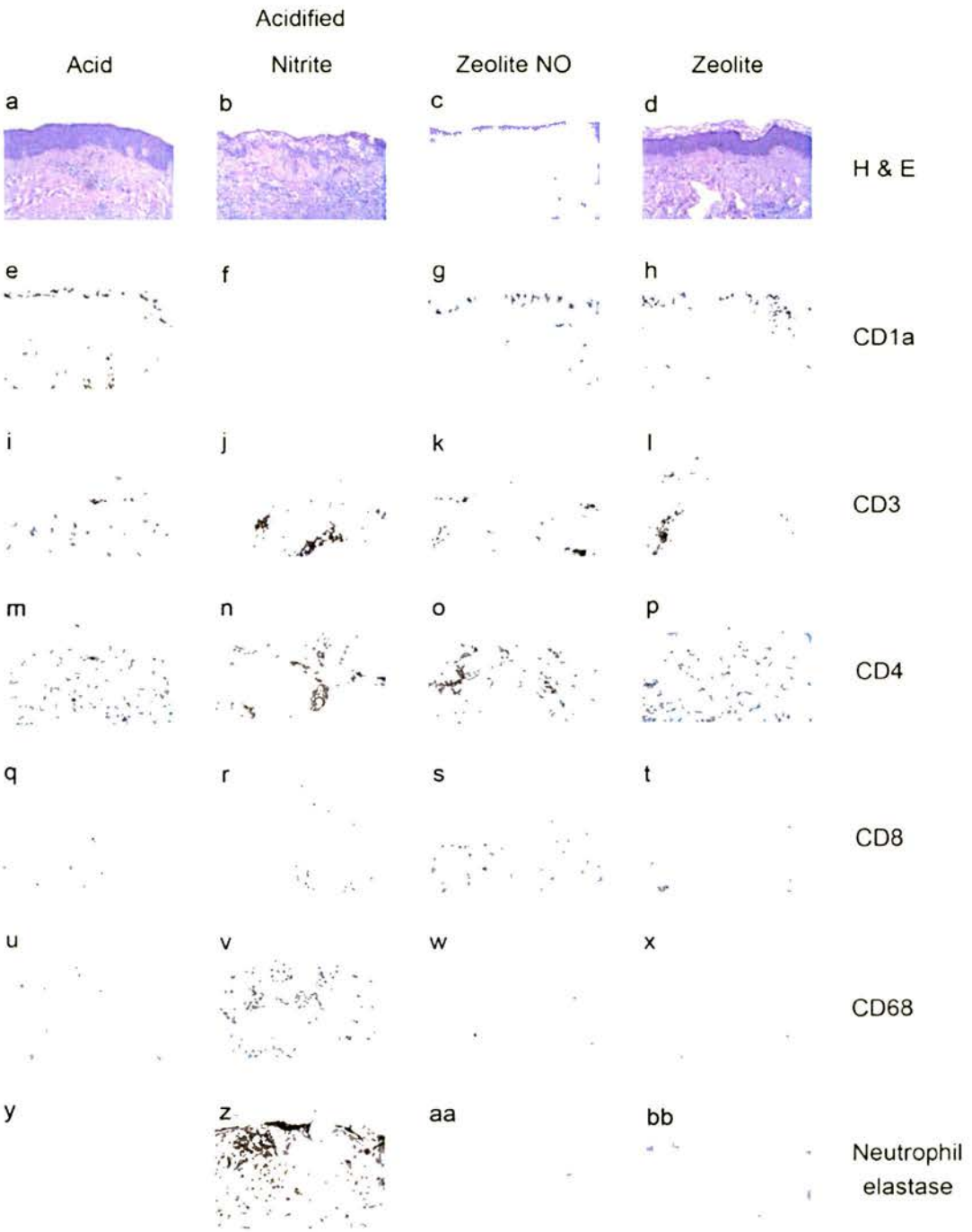


Figure 3.4 *H & E and immunohistochemical staining of skin following topical application of 33% Ze-NO, 5% acidified NO_2^- and controls (x40)*

3.4.3. Epidermal results

3.4.3.1.Acidified NO_2^-, but not Ze-NO, causes infiltration of macrophages and neutrophils into the epidermis

A significant increase in macrophages and neutrophils was seen in the epidermis, following the application of topical acidified NO_2^- compared with it's own control (2% ascorbic acid), zeolite control and Ze-NO (figure 3.4 & 3.5).

3.4.3.2.Acidified NO_2^- but not Ze-NO, reduces Langerhans cells in the epidermis

A highly significant loss of Langerhans cells from the epidermis was seen following topical application of acidified NO_2^-. This finding was significantly different to that seen with the ascorbic acid control, Ze-NO, and Zeolite alone (figure 3.4 and 3.5).

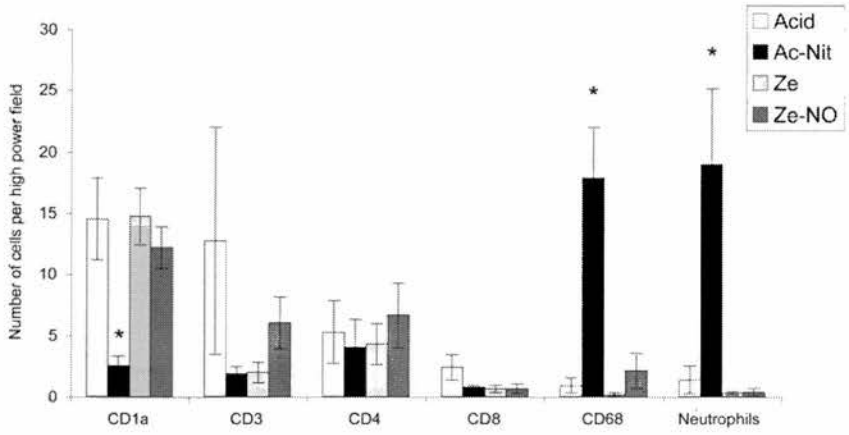


Figure 3.5 *Inflammatory cells in the epidermis following application of 33% Ze-NO, 5% acidified NO_2^- and controls, quantified by immunohistochemical staining (n=6, ±SEM)*

** acidified NO_2^- compared with acid control, zeolite control and Ze-NO $p<0.05$*

3.4.4. Dermal results

3.4.4.1.Both acidified NO_2^- and Ze-NO result in a moderate increase in dermal T cells

A significant increase in dermal CD3, 4 and 8 positive T cells was seen following topical application of acidified NO_2^-, a less marked but significant increase was seen in CD 4 positive T cells following topical application of Ze-NO (figure 3.4 & 3.7). Suction blister fluid in Ze-NO treated skin had significantly increased IFN γ when compared with control blister fluid (mean control 0.1±0.07pg/mg protein, mean IFN γ 0.6±0.4pg/mg protein, figure 3.6), there was no detectable IL 4 (data not shown). While CD4 can be expressed on macrophages, the finding of elevated IFN γ in association with CD3 and CD4 positive cells strongly suggests the presence of infiltrating T lymphocytes of the Th1 family.

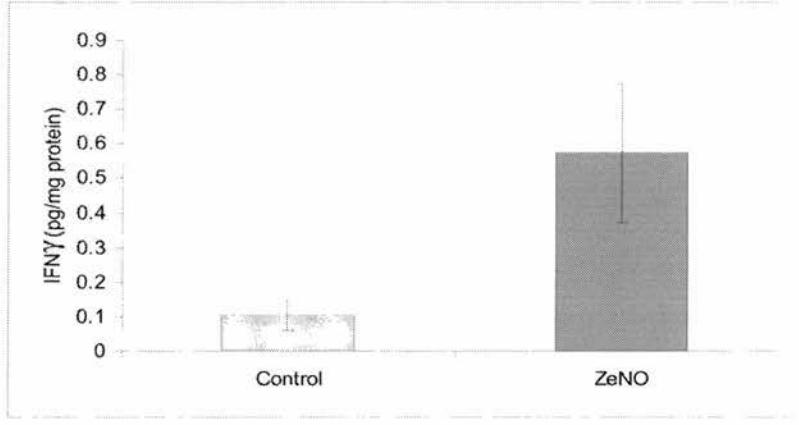


Figure 3.6 *ELISA analysis of epidermal suction blister fluid, data shows IFN γ concentration in epidermal suction blister fluid after correction for protein concentration*

n=4, mean ± SEM, control 0.10pg/mg protein, Ze-NO 0.57pg/mg protein, $p<0.05$

3.4.4.2.Acidified NO_2^- causes infiltration of macrophages and neutrophils into the dermis

A significant increase in macrophages and neutrophils was seen in the dermis following topical acidified NO_2^- application in comparison with acid control and both zeolite preparations (figure 3.4 & 3.7).

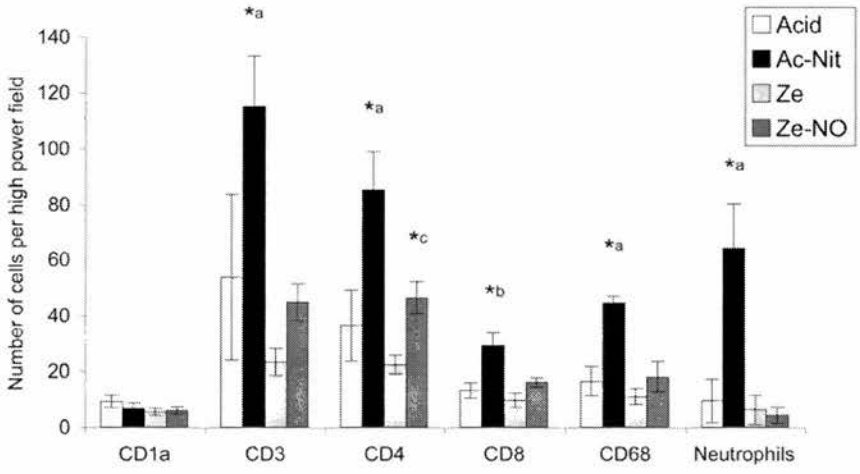


Figure 3.7 *Inflammatory cells in the dermis following application of 33% Ze-NO, 5% acidified NO_2^- and controls, quantified by immunohistochemical staining*

**a acidified NO_2^- compared with all other preparations, $p<0.05$. *b acidified NO_2^- compared with acid and zeolite controls, $p<0.05$. *c Ze-NO compared with zeolite control, $p<0.05$.*

3.5. Discussion

Acidified NO_2^- produced by the combination of NO_2^- and ascorbic acid has potent inflammatory effects on human skin *in vivo* as Ormerod initially showed(Ormerod et al., 1999a), and as has been confirmed in these experiments. I suggest that acidified NO_2^- is not an ideal topical NO donor, as in addition to NO it also releases a number of other potentially pro-inflammatory mediators.

The chemically simplest form of acidified NO_2^- is sodium NO_2^- to which hydrochloric acid has been added. This gives rise to NO as well as to other nitrogen oxides (figure 3.8).

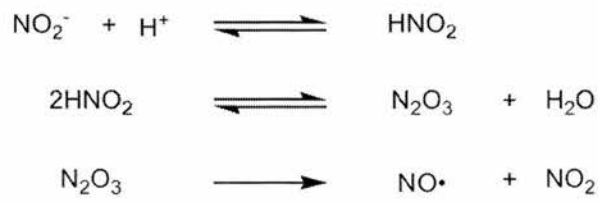


Figure 3.8 *Acidified NO_2^- is formed by mixing sodium NO_2^- with hydrochloric acid which releases NO*

The weak acid ascorbic acid may seem to the non-chemist like an ideal candidate for a formulation of acidified NO_2^-, but the reaction between ascorbic acid and sodium NO_2^-, which also gives NO, is different to that described above(Dahn et al., 1960) (figure 3.8). When ascorbic acid and NO_2^- are mixed, the N_2O_3, formed from NO_2^- and H^+, is reduced with subsequent release of NO and ascorbyl radicals (figure 3.9).

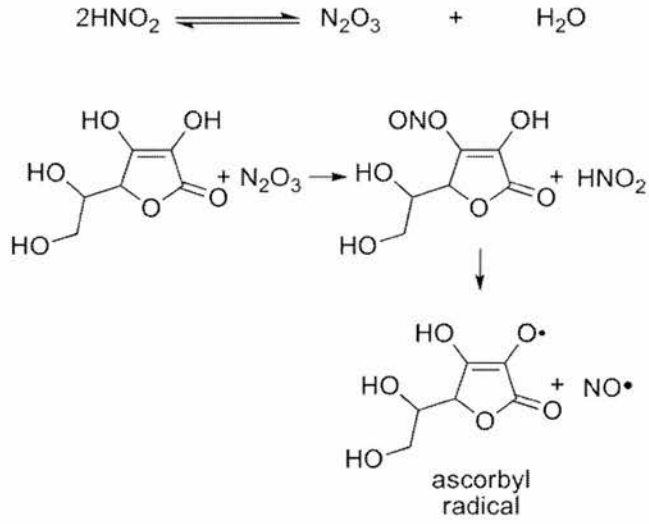


Figure 3.9 *Ascorbic acid and NO_2^- when placed on the skin form N_2O_3 from NO_2^- and H^+, this is reduced with subsequent release of NO and ascorbyl radicals*

I suggest it is these ascorbyl radicals, and additional species formed downstream of them, which give the NO_2^--ascorbic acid mix such potent pro-inflammatory properties. We hypothesise that the ascorbyl radicals within the cream diffuse into the skin, alternatively endogenous ascorbate may also form radicals within the skin. In addition to being an inflammatory stimulus itself, the ascorbyl radical has the

potential to form further pro-inflammatory species: a) in the presence of oxygen, pro-inflammatory hydrogen peroxide is formed (figure 3.10).

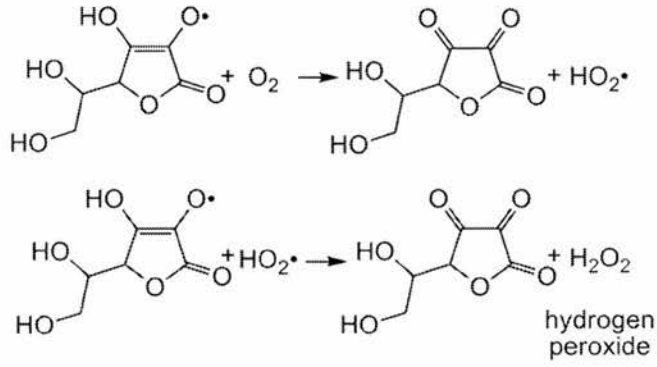


Figure 3.10 *In the presence of oxygen the ascorbyl radical has the potential to form hydrogen peroxide*

b) The ascorbyl radical may react with cysteine or tyrosine to give cysteinyl or tyrosyl radicals, c) the ascorbyl radical may also react with lipids in cell membranes producing a pro-inflammatory hydroxyl radical (figure 3.11).

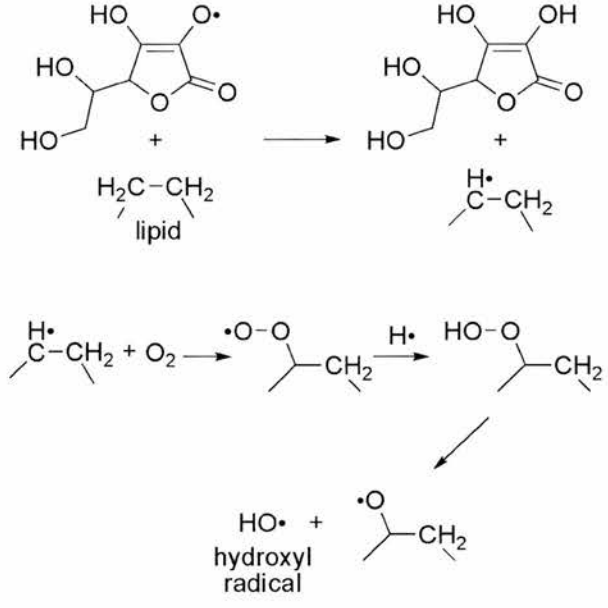


Figure 3.11 *The ascorbyl radical may react with lipids in cell membranes producing a pro-inflammatory hydroxyl radical*

In contrast to acidified NO_2^-, Ze-NO produces effects caused directly by NO itself. Zeolite based compounds are already used clinically as MRI gastrointestinal contrast

agents and as clotting enhancers. Zeolite-A (LTA) consists of alternating SiO_4 and AlO_4 tetrahedra that share corners to produce an open framework (figure 1.14). It has a natural affinity for NO which is bound within the framework, and NO is released (delivered) from this stable storage material in biologically relevant amounts on contact with water. Ze-NO has anti-thrombotic properties, by inhibiting platelet aggregation and adhesion in plasma(Wheatley et al., 2006). The release kinetics of NO from zeolites can be tailored by altering the type and number of metal cations in the structure, allowing NO delivery patterns to be modulated to specific clinical requirements. In preliminary experiments looking at different formulations of Ze-NO it was found that using manganese (Mn) or cobalt (Co) within the zeolite structure led to a more prolonged release of NO than other cations. Mn-Ze-NO was selected for use in the main experiments as cobalt may induce an allergic contact dermatitis.

The Langerhans' cell number was shown to be greatly decreased in the epidermis of skin treated with acidified NO_2^-, compared to that treated with Ze-NO or vehicle controls (figure 3.5). Langerhans' cells are bone marrow-derived antigen-processing and presenting cells that are involved in a variety of T cell responses. They are the primary cells in the epidermis that are responsible for the recognition, uptake, processing, and presentation of soluble antigen and haptens to sensitised T lymphocytes. Langerhans cells in the epidermis can ingest and process antigens, they then migrate to the draining lymph node. Activated Langerhans' cells that have been induced to migrate after contact with antigen are potent stimulators of naïve T cells. I interpret the marked reduction of Langerhans' cells in the epidermis of skin treated with acidified NO_2^- as a further indication of the inflammatory properties of this topical preparation, it is postulated that acidified NO_2^- induces a massive migration of Langerhans' cells to their draining lymph nodes. An alternative explanation to this reduction in Langerhans' cell number, is that topical acidified NO_2^- is responsible for inducing such an intense inflammatory reaction that the epidermis itself is disrupted, and hence Langerhans' cells are simply shed within the epidermis that is lost. Certainly some epidermal disruption can be seen both clinically and histologically in the sections of skin treated with acidified NO_2^- (figure 3.3 & 3.4). Langerhans' cell

migration is also a characteristic finding following UV radiation and a component of UV induced immunosuppression. NO has been shown to be a mediator of Langerhans cell migration in UV radiation induced immunosupression to nickel contact hypersensitivity(Kuchel et al., 2003). In the experiments detailed in this chapter, Ze-NO produced a small, but non-significant fall in Langerhans' cell density. The degree of reduction in Langerhans' cells is almost certainly dose dependent, as Kuchel was using 5 MED's of solar simulated radiation(Kuchel et al., 2003), while the erythema (and thus NO delivery) produced by our Ze-NO donor was equivalent to 0.75-1MED.

The majority of significant inflammatory cell infiltration occurred in the dermis as opposed to the epidermis. Ze-NO induced a mild Th1 infiltrate whereas acidified NO_2^- produced a much greater mixed infiltrate of CD3, 4, 8, and 68 positive cells and neutrophils. The charge neutrality of NO facilitates its free diffusibility through aqueous solutions and across membranes. NO released from Ze-NO can thus diffuse across the epidermis to the dermis to exert its biological effects, where it can be oxidised to form aqueous NO_2^-, an active storage form of NO which serves to prolong its activity.

The data presented in this chapter give a clear indication that NO from Ze-NO leads to a CD4 positive lymphocyte infiltration of the skin (figure 3.4 & 3.7), with IFN γ cytokine secretion typical of Th1 lymphocytes (figure 3.6). Great care has been taken in these experiments to ensure that the amounts of NO delivered to the skin by Ze-NO match that of acidified NO_2^-, this comparability was ensured by measuring both NO donor induced changes in dermal blood flow and also dermal NO_2^- concentration by microdialysis (figure 3.1 & 3.2). Data obtained from these studies would suggest that the biological effects of NO itself are more specific and limited than was previously demonstrated by the generalised inflammation produced by a mix of NO_2^- and ascorbate.

Psoriasis is characterised by a CD4 positive T lymphocyte infiltration to the upper dermis(Baker and Fry, 1992) with a predominantly Th1 pattern of cytokine secretion, and by hyperproliferative, poorly differentiated keratinocytes in the epidermis. The iNOS enzyme is consistently found in the dermis and basal epidermis of psoriatic plaques(Bruch-Gerharz et al., 1996;Rowe et al., 1994), and the characteristic erythema seen clinically, indicates vasodilatation and thus presumably biologically significant NO production. Measurements with a chemiluminescence meter from the surface of psoriatic plaques show elevated NO release(Ormerod et al., 1997), and serum NO_2^-, an indicator of systemic NO production, is elevated in psoriatics with active disease(Tekin et al., 2006). However, it has been suggested more recently that the role of NO in psoriasis is more complex than can be explained simply by an upregulation of iNOS. The effects of NO are frequently dose dependent, but in a non-linear fashion with opposing effects seen at low and high concentrations. Although iNOS is upregulated in psoriatic plaques, the generation of NO is maintained at relatively low concentrations due to a concomitant upregulation of arginase 1. Arginase 1 competes for the common substrate L-arginine (figure 1.3), thus maintaining NO at relatively low levels despite the observed upregulation of iNOS. It has been shown that relatively low levels of NO promote keratinocyte proliferation, consistent with the histological picture of psoriasis, whereas higher levels of NO arrest cell proliferation and initiate differentiation(Krischel et al., 1998).

The pleiotropic nature of NO in influencing keratinocyte proliferation is also observed when studying T cells. Niedbala *et al.* have shown that NO, in a low concentration, up regulates IL-12Rβ2 expression on naïve T cells, which allows for induction of Th1 cells but has no influence on Th2 cell differentiation(Niedbala et al., 2006). The selective enhancement of Th1 differentiation by NO is dependent on it being at low concentration, with high NO concentrations being cytotoxic to this T cell population. The data obtained from the experiments in this chapter are consistent with this observation, and provide early evidence to show Th1 lymphocyte infiltration induced by relatively low concentrations of NO *in vivo* in man. The Ze-NO preparation used in the experiments detailed in this chapter produced a just

perceptible erythema, consistent with the erythema observed after one MED UVB, and therefore a biologically relevant and relatively low concentration of NO.

In summary the data provided in this chapter suggest that Ze-NO is an effective NO donor in man, and does not exhibit the dramatic pro-inflammatory effects which were previously attributed to NO. The data presented concurs with Kolb-Bachofen's hypothesis that iNOS activity in psoriatic plaques produces a relatively slight elevation in NO levels owing to substrate competition by arginase 1. A moderate Th1 cell response is induced by these low concentrations of NO, which is in keeping with current views on the role of NO in the regulation of T cell functions(Niedbala et al., 2006). It has been shown that an ascorbic acid-NO_2^- combination is a potent pro-inflammatory mix, a number of mediators in addition to NO are potentially active in this process (figures 3.9-3.11). Ze-NO has been demonstrated to be an effective topical NO donor that releases physiologically relevant concentrations of NO. It has been vital to find such an NO donor before I can progress to the investigations planned for the remainder of my programme of clinical research.

CHAPTER 4

NO STORES IN HUMAN SKIN

4. CHAPTER 4 - NO STORES IN HUMAN SKIN

4.1. Introduction

Three NOS enzymes have been isolated, these are responsible for enzyme-dependent NO formation(Moncada and Higgs, 1993). Two constitutive and one inducible NOS isoenzyme exist, each of which has been isolated in human skin(Baudouin and Tachon, 1996;Becherel et al., 1994;Bull et al., 1996;Romero-Graillet et al., 1996;Sakai et al., 1996;Wang et al., 1996). The $t^{1/2}$ of NO (0.05-1.18milliseconds)(Borland, 1991;Liu et al., 1998;Vaughn et al., 2000) is very short, and thus it was initially regarded as having actions only in close proximity to its site of production. It is now accepted that NO can form additional bioactive stable carriers/donors in the blood and tissues by reacting with SH groups to form RSNOs(Stamler et al., 1992b;Stamler et al., 1992a). These regulate proteins by S-nitrosation of specific cysteine residues(Hess et al., 2001). A major proportion of endogenous NO is also inactivated by oxidation to NO_2^- and the more stable NO_3^-.

It has recently become possible to quantify NO-related products in biological samples, using a technique of gas-phase chemiluminescence(Feelisch et al., 2002). Paunel *et al.,* have used this technique to quantify NO-related products in *ex-vivo* full thickness human skin specimens(Paunel et al., 2005). Paunel *et al.,* have also shown evidence to suggest that UVA irradiation induces rapid photolysis of RSNO and NO_2^-. The photolysis of NO-related products in human skin leads to high output enzyme-independent NO production, which occurs within 20 minutes after the onset of UV exposure(Paunel et al., 2005).

NO reduces UV induced apoptosis in mouse dermis and epidermis(Weller et al., 2003), and a protective role of iNOS has also been shown in endothelial cells *in vitro* following UVA irradiation(Suschek et al., 1999). It has been hypothesised that the rapid UVR-induced, enzyme-independent release of NO from NO-related products in human skin, is biologically important. This photolytic release from NO stores

potentially 'bridges the gap' between UV challenge and the enzymatic up-regulation and activity of iNOS, which is maximal 8-10 hours post irradiation(Paunel et al., 2005) (figure 1.13).

4.2. Aims

The aim of this study was:

- To quantify NO-related products in human skin *in vivo*:
    - In sweat on the skin surface
    - In epidermis
    - In superficial vascular dermis
- To determine *in vivo* the extent to which NO release can be demonstrated during UVA irradiation.

4.3. Methods

All methods common to more than one study are detailed in Chapter 2.

4.3.1. Study volunteers

Three individual studies were performed, each using healthy volunteers.

- Quantification of NO-related products in epidermal suction blisters and blister fluid - ten subjects, age range 22-41 years, three male.
- Quantification of NO-related products in human sweat, plasma and saliva - ten subjects, age range 20-29 years, six male.
- Quantification of NO-related products in the superficial vascular dermis - eighteen subjects, fourteen of these also underwent UVA irradiation during cutaneous microdialysis and eight had plasma NO_2^- measured at the time of their microdialysis. Age range 20-50 years, four male.

4.3.2. Saliva and Plasma Collection

One hour prior to commencing sweat collection and cutaneous microdialysis, samples of venous blood (5ml) were taken from each subject. Samples of saliva (3ml) were also taken from subjects attending for the sweat study. Blood and saliva samples were stored in NEM/EDTA solution and centrifuged immediately at 1300g for eight minutes. Supernatants were collected and stored at $^{-}70^{0}$C for a maximum of three days before analysis.

4.3.3. Sweat Collection

An anaerobic method of sweat collection was used which allows for the collection of relatively large volumes of sweat with minimal epidermal contamination(Boysen et al., 1984). Subjects were taken to the Edinburgh commonwealth pool sauna in order to perform adequate sweat collection at rest. Sweat samples were collected into eppendorfs containing NEM/EDTA and stored at $^{-}70^{0}$C for a maximum of three days before analysis.

Figure 4.1 *Method of anaerobic sweat collection from human skin*

4.3.4. Cutaneous microdialysis and UVA exposure

Dermal microdialysis catheters were used to measure NO concentrations within the superficial dermis and to quantify NO release following UV exposure. Two sites, one on the volar aspect of the forearm and the second on the flexor aspect of the upper arm, were anaesthetised. Four microdialysis catheters were inserted into the superficial dermis parallel with the skin surface at each of the two anaesthetised sites. Normal saline was dialysed continuously through two catheters at each site, noradrenaline was dialysed continuously through the two remaining catheters at each site. Dialysate was collected continuously in 15 minute aliquots. Following insertion of the microdialysis catheters dialysate was collected for 30 minutes prior to the onset of UVA exposure. Catheters in the volar forearm were irradiated with UVA whereas those in the flexor aspect of the upper arm remained unirradiated.

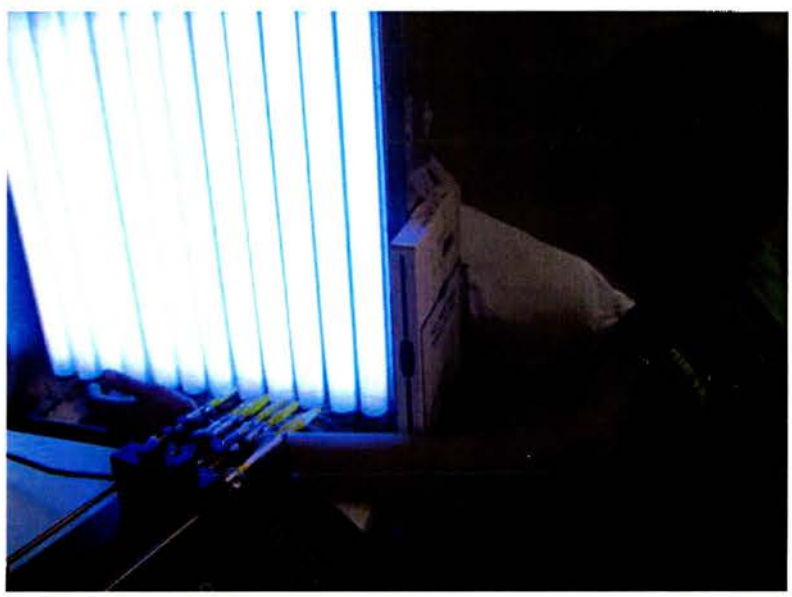


Figure 4.2 *Cutaneous microdialysis during UV exposure*

4.3.5. UV source

The UVA source used was a bespoke cabinet containing a panel of 12 bulbs (Philips TLK 40W/10R R-UVA, Philips Hamburg, Germany) emitting a UVA spectrum 350-400nm, peak 360nm, 10mW/cm^2 (figure 1.9). Volunteers were positioned so that the

skin to be irradiated was parallel to the light source and irradiated for 60 minutes ($60J/cm^2$).

4.4. Results

4.4.1. The concentration of total NO-related products in human saliva is ten times greater than that in the superficial vascular dermis, plasma and sweat

The concentration of total NO-related products in saliva (267 ± 194.67μM) was significantly greater than that found in human superficial vascular dermis (12 ± 5.97μM), plasma (27 ± 16.28μM) and sweat (22 ± 9.34μM) (figure 4.3, table 4.1).

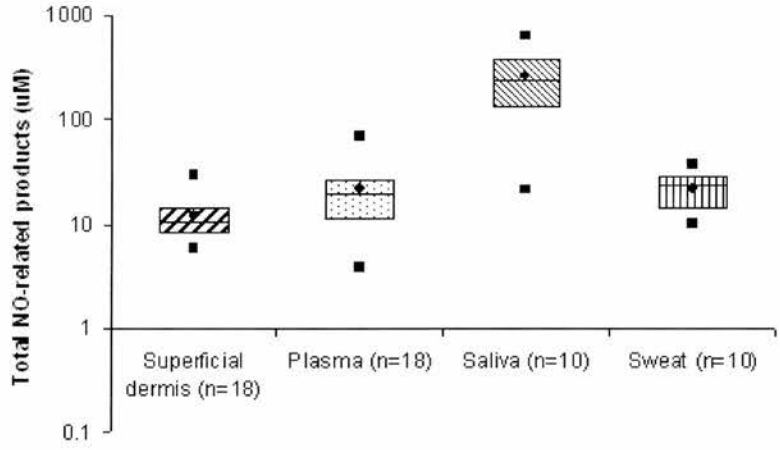


Figure 4.3 *Total NO-related products in human superficial vascular dermis, plasma, saliva and sweat*

Boxplots represent median and interquartile range, ▪ maximum and minimum values, • mean.

These findings are consistent with those of Lundberg *et al.,* who found the mean salivary NO_3^- and NO_2^- concentrations of nine subjects to be 0.19mM and 104μM respectively. The same nine subjects had plasma NO_3^- and NO_2^- concentrations of 30μM and 123nM respectively(Lundberg and Govoni, 2004) (table 4.1). Ingested NO_3^- is absorbed from the gastrointestinal tract into the blood stream where it mixes

with endogenously synthesized NO_3^-. Most NO_3^- is excreted in urine but some is secreted in saliva, sweat and the gastrointestinal tract(Lundberg and Govoni, 2004). The high salivary concentrations of total NO-related products are explained by this enterosalivary circulation (figure 1.6), in which up to 25% of plasma NO_3^- is taken up by the salivary glands and secreted with saliva(Spiegelhalder et al., 1976). Microorganisms such as those found on the tongue surface can anaerobically reduce NO_3^- to NO_2^- (Lundberg and Govoni, 2004). On passing to the stomach, acidification of NO_2^- releases large amounts of NO and other RNSs.

4.4.2. NO_3^- accounts for the majority of the total NO-related products in saliva, plasma, sweat and epidermis.

In all biological samples in which individual NO-related products were quantified NO_3^- was found to be the predominant species. Sixty to seventy-five percent of plasma, saliva and sweat total NO-related products was NO_3^-, with the majority of the remainder being NO_2^- (figure 4.4).

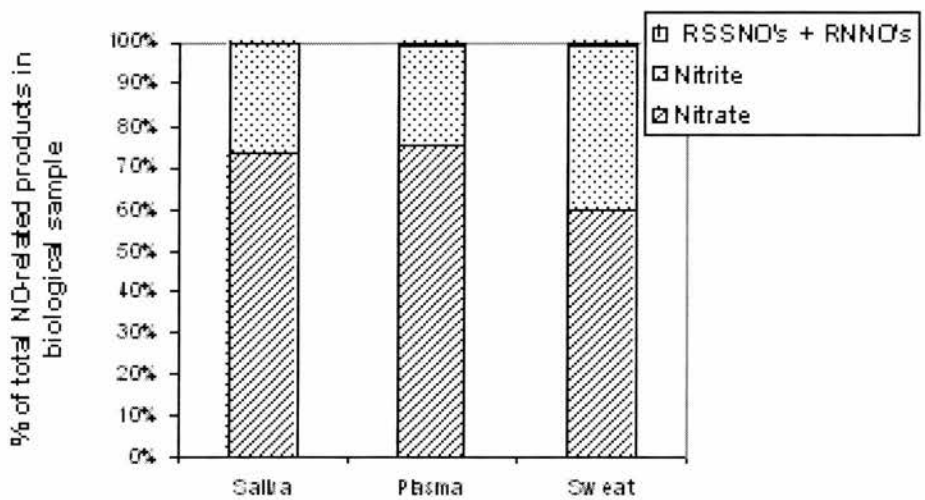


Figure 4.4 *The proportion of individual NO-related products found in human saliva, plasma and sweat*

NO_3^- is the predominant NO-related product contributing to 60-75% of the total, NO_2^- makes up the majority of the remainder (24-39%), negligible amounts of RS/NNOs were found (0-1%).

A similar proportional split between NO_3^- and NO_2^- to that seen in saliva, plasma and sweat, was observed when measuring individual NO-related products in epidermal suction blisters and blister fluid (figure 4.5).

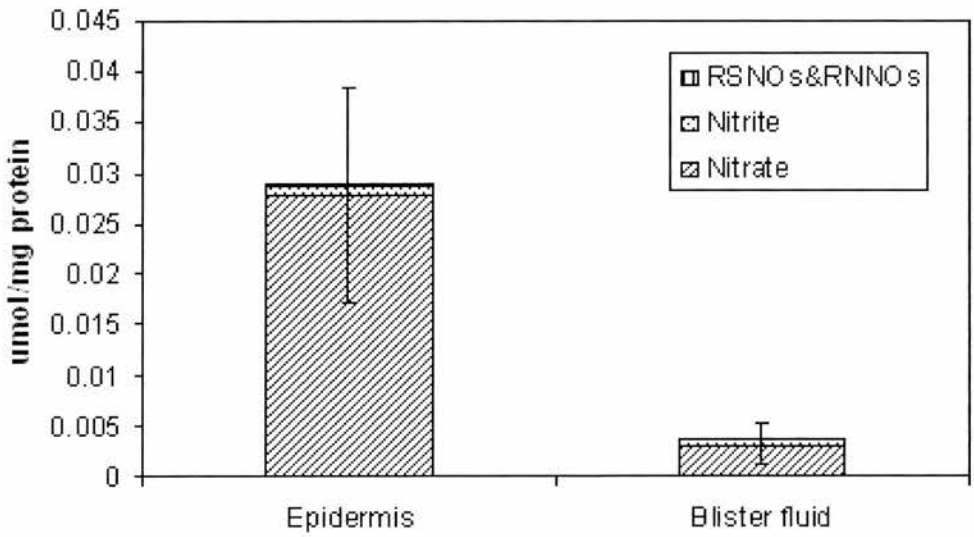


Figure 4.5 *Suction blister fluid contains negligible NO-related products compared to epidermis*

Mean total NO-related products epidermis 0.03 ± 0.03μmol/mg protein, mean total NO-related products suction blister fluid 0.004 ± 0.007μmol/mg protein, n=10, error bars = SEM.

Epidermal suction blister data is displayed as μmol/mg protein and therefore not directly comparable to the other biological species measured. Paunel *et al.,* are the only other group to have measured NO-related products in human skin. In contrast to my data they used *ex vivo* full thickness frozen sections(Paunel et al., 2005). Following adjustment of my data to μM NO_2^- with equivalent protein concentrations, my results are comparable with those of Paunel *et al.*, (table 4.1). In addition to analysing epidermis from the roof of suction blisters, blister fluid was also examined for the concentration of NO-related products. In contrast to the epidermis, epidermal suction blister fluid was high in protein content but contained negligible NO-related products (figure 4.5).

μM Measured using NO_2^- standards	Total NO-related products	NO_3^-	NO_2^-	RSNOs&RNNOs
Saliva (n=10)	267.13	197.38	69.55	0.21
Plasma (n=10)	27.38	20.60	6.59	0.20
Sweat (n=10)	21.97	13.13	8.66	0.19
Superficial dermis (NaCL, n=18)	12.31	not measured	Not measured	not measured
Saliva(Lundberg and Govoni, 2004)	*294.03*	*190.00*	*104.00*	*0.03*
Plasma(Lundberg and Govoni, 2004)	*30.13*	*30.00*	*0.12*	*0.01*
Sweat(Weller et al., 1996)	*43.12*	*39.70*	*3.42*	*not measured*

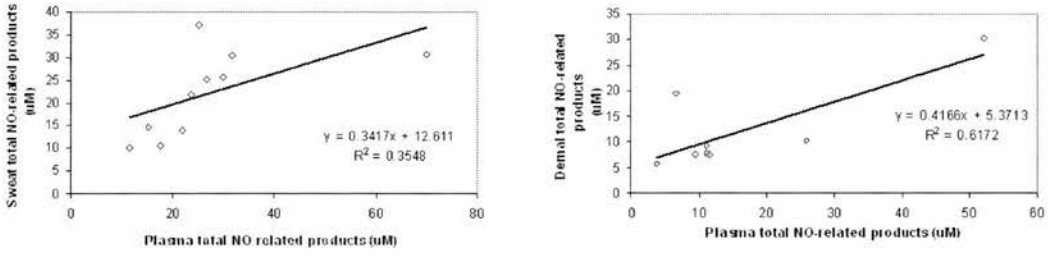
Measured as μmols NO_2^-/mg protein	Total NO-related products	NO_3^-	NO_2^-	RSNOs&RNNOs
Epidermis	0.0289	0.0278	0.0008	0.0003
Epidermal suction blister fluid	0.0036	0.0031	0.0005	0
Epidermis adjusted to compare with Paunel data (μM NO_2^-)		278.15	8.37	2.89
Epidermis & dermis (μM NO_2^-)(Paunel et al., 2005)		*82.4*	*5.1*	*2.6*

Table 4.1 *Summary of the concentration of NO-related products at different sites and comparison with published data (shown in italics)*

4.4.3. The concentration of total NO-related products in human plasma corresponds to one third of the concentration of total NO-related products in sweat and two thirds of the concentration of total NO-related products of the superficial dermis.

The concentration of total NO-related products in human sweat and plasma was measured in ten subjects, and of the superficial vascular dermis and plasma in 18 subjects.

Regression analysis suggests that the concentration of NO-related products in plasma is linked to the concentration of NO-related products found in sweat ($R^2 = 0.34$, figure 4.6a) and in the superficial dermis ($R^2 = 0.62$, figure 4.6b).



a) sweat NO-related products *b) dermal NO-related products*

Figure 4.6 *Relationship between plasma NO-related products and a) sweat NO-related products, b) dermal NO-related products*

Paunel *et al.* have demonstrated a correlation between the concentration of NO_2^- and RSNOs in the skin and the formation of NO from the skin within 20 minutes of exposure to UVA(Paunel et al., 2005). Taking the findings of Paunel *et al.,* into consideration I hypothesise that plasma NO-related products may partly determine an individual's response to UVA exposure.

4.4.4. The concentration of total NO-related products vary between individuals

Cutaneous microdialysis was used to quantify aqueous NO-related products in the superficial vascular dermis *in vivo*. Over a 90 minute period of sampling, using normal saline as dialysate fluid, the concentration of NO-related products showed minimal variation within subjects (figure 4.7). There was however an approximately four fold inter-individual variation in total NO-related products of the superficial dermis (median 10.49μM, IQR 6.3μM, figure 4.3 and 4.7), which is consistent with the known wide variation in levels of tissue NO-related products observed following NO_2^- and NO_3^- intake(Gladwin et al., 2005).

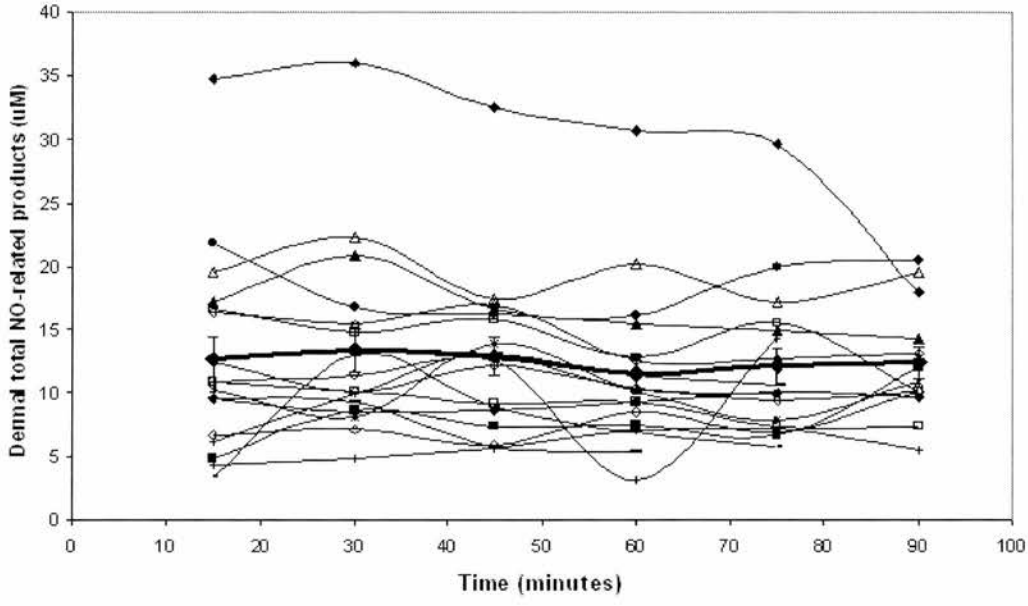


Figure 4.7 *Aqueous NO-related products in the superficial dermis over a 90 minute period of sampling*

Mean dermal concentration of NO-related products is represented by ♦.

Mean 12.31 ± 5.97μM, error bars = SEM (n=18)

4.4.5. UVA irradiation increases the yield of aqueous NO-related products from the superficial vascular dermis. This is reduced by local vasoconstriction

The yield of aqueous NO-related products from the superficial dermis was lower on dialysing with noradrenaline than normal saline. UVA irradiation significantly increased the yield of total NO-related products from the dermis after 30 minutes when normal saline was used as a dialysate, this was abrogated when noradrenaline was used as a dialysate. Mean total NO-related products of the superficial dermis during irradiation: unirradiated normal saline 12.49 ± 0.64μM, irradiated normal saline 15.50 ± 0.93μM, unirradiated noradrenaline 11.12 ± 1.02μM, irradiated noradrenaline 12.45 ± 0.65μM (figure 4.8).

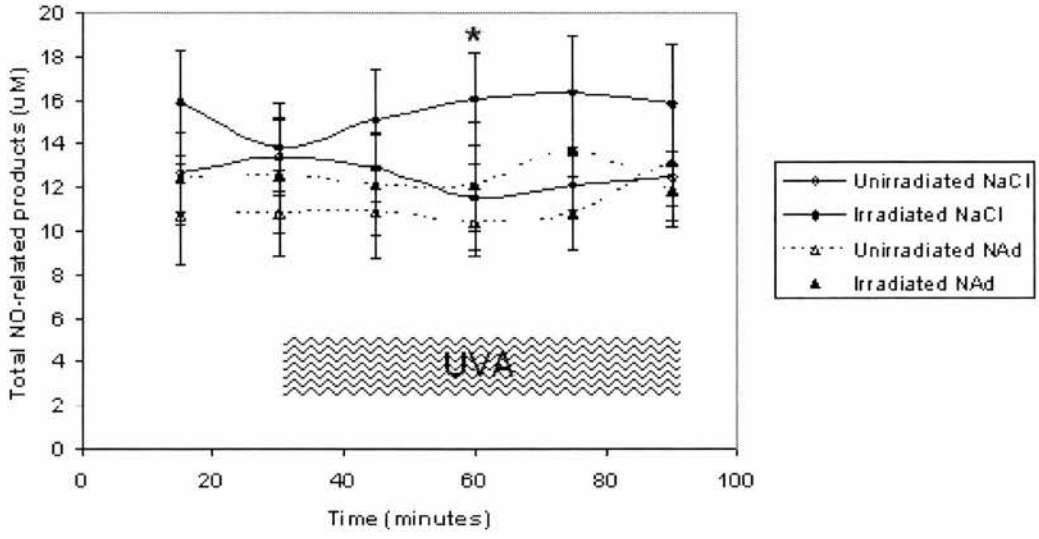


Figure 4.8 *NO-related products in the superficial dermis following UVA exposure, comparison between normal saline and noradrenaline as dialysate*

*Unirradiated normal saline (n=18), irradiated normal saline (n=14), unirradiated noradrenaline (n=12), irradiated noradrenaline (n=11). * $p<0.05$ on comparison of unirradiated with irradiated using normal saline as dialysate, error bars = SEM.*

The cumulative concentration of total NO-related products during the period of irradiation was significantly higher in the irradiated than unirradiated skin when dialysed with normal saline, but there was no significant difference when noradrenaline was used as dialysate (figure 4.9). These findings suggest that the superficial dermal vasculature is important in the delivery of NO and NO-related products to the skin following UVA exposure.

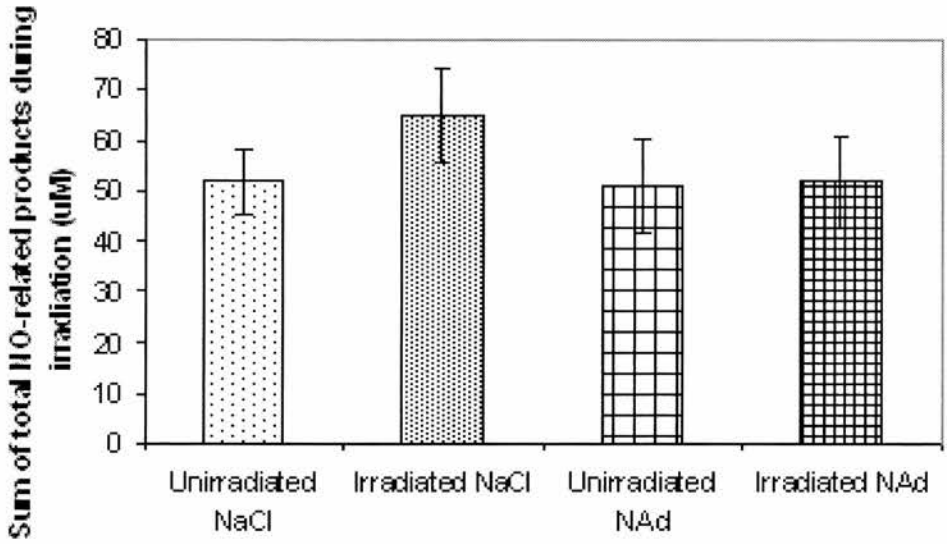


Figure 4.9 *Sum of NO-related products in the superficial dermis during period of UVA exposure, comparison between normal saline and noradrenaline as dialysate*

*Unirradiated normal saline (n=18), irradiated normal saline (n=14), unirradiated noradrenaline (n=12), irradiated noradrenaline (n=11). * $p<0.05$ on comparison of unirradiated with irradiated using NaCl as dialysate, error bars = SEM.*

4.4.6. UVA exposure induces NO release within the superficial vascular dermis which is maximal 30 minutes after the onset of exposure

When normal saline is used as a dialysate there is a significant increase in aqueous NO-related products 30 minutes after the onset of UVA irradiation (30J/cm^2, figure 4.10). These findings are consistent with the data of Paunel *et al.,* who have demonstrated NO release from *ex-vivo* human skin specimens maximal 20 minutes after the onset of UVA exposure (40J/cm^2)(Paunel et al., 2005). Both the *in vivo* results of this experiment, and the *ex vivo* results of Paunel *et al.,* are consistent, they suggest that UVA exposure results in photolysis of NO stores in human skin and the rapid release of NO. This process is maximal 20-30 minutes after the onset of UV exposure, corresponding to 30-40J/cm^2.

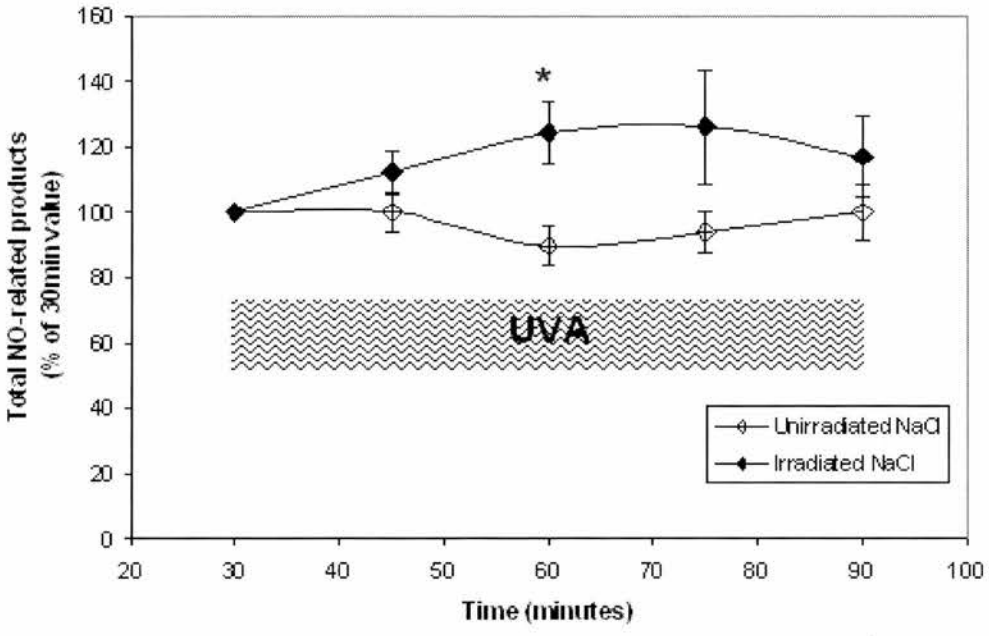


Figure 4.10 *NO-related products in the superficial dermis following UVA exposure using normal saline as dialysate*

*Data normalized to 30 minute value, n=14, error bars = SEM, * $p<0.05$.*

4.5. Discussion

It is widely accepted that NO-related products in the form of NO_3^-, NO_2^- and RSNOs are important storage forms of NO, the latter two offering potential sources of recyclable bioactive NO(Lundberg and Govoni, 2004;Rassaf et al., 2004). Nitrite has been shown to act as a signalling molecule in its own right(Bryan et al., 2005), and can also provide a bioavailable pool of NO during periods of hypoxia and ischaemia(Wink et al., 1996b;Zweier et al., 1995). RSNOs have also been implicated as important storage and transport systems for NO. Low molecular weight RSNOs exert NO-like activity *in vivo*(Ignarro et al., 1981) and circulating RSNOs can release NO when required(Rassaf et al., 2004). The relative importance of NO_2^- and RSNOs in NO bioactivity continues to be debated, in part fuelled by the different methods used to quantify these species in blood and tissues. A wide range of basal NO_2^-

concentrations in human plasma have been reported, from undetectable to 26μmol/L, but the majority fall in the nmol/L range(Gorenflo et al., 2001;Lundberg and Govoni, 2004;Meulemans and Delsenne, 1994;Moriel et al., 2001;Pelletier et al., 2006;Rassaf et al., 2002a;Rocks et al., 2005). A similar variation exists for documented quantities of RSNOs in human plasma from undetectable to 930nMol/L(Rocks et al., 2005). The principle explanation for the continued debate regarding the contribution of individual NO-related products to normal physiology and pathophysiology lies in the lack of sensitive assays for their detection and the influence that proteins, varying redox conditions and trace contamination with NO_2^- may have on these assays.

Chemiluminescence-based detection is a method of quantification of NO-related products which is simple, sensitive, reproducible and suitable for the analysis of a high number of samples within a short period of time(Marley et al., 2000), it was thus ideal for the purpose of the experiments presented in this chapter. Nanomolar levels in basal NO_2^- concentration have been found in human plasma using the same chemiluminescence method as was employed in these experiments. Kleinbongard *et al.*, compared three different detection methods including chemiluminescence, a high-pressure liquid chromatography (HPLC) technique and flow injection analysis, and demonstrated similar concentrations of NO-related products across species with all three methods(Kleinbongard et al., 2003).

I have demonstrated that in human epidermis and in sweat on the skin surface NO_3^- is the predominant NO-related products, followed by NO_2^-, with only very low concentrations of RSNOs being present. Both NO_3^- and NO_2^- are available from exogenous and endogenous sources. Green leafy vegetables make up 60-80% of the daily NO_3^- intake of those on a typical western diet(Ysart et al., 1999) whereas NO_2^- is used as a preservative for meat and fish(Lundberg and Govoni, 2004). The main endogenous source of NO_3^- and NO_2^- is the L-arginine – NO pathway which is constitutively active in all cell types in the body. L-arginine is converted to NO by

iNOS, an enzyme which is upregulated in human skin 8-10 hours after UV exposure(Kuhn et al., 1998). Nitrite can also be generated from commensal bacteria in the digestive system by NO_3^- reduction. An enterosalivary recirculation pathway exists, with 25% of all circulating NO_3^- being taken up by the salivary glands and secreted in saliva. The oral cavity contains large numbers of NO_3^--reducing bacteria, with the result that saliva is the main source of plasma NO_2^-(Benjamin et al., 1994). Lundberg *et al.*, have demonstrated that dietary NO_3^- ingestion influences both plasma NO_3^- and NO_2^- but not RSNO levels(Lundberg and Govoni, 2004). Interpretation of the data from the experiments presented in this chapter suggests that the concentration of NO-related products in an individual's plasma will influence the concentration of NO-related products found in their sweat and superficial vascular dermis. I hypothesise that ultimately dietary NO_3^- may offer a source for manipulation of cutaneous NO-related products.

Historically, evidence for the existence of stored forms of NO dates back to experiments performed in the 1950s on the relaxant effect of light on vascular smooth muscle(Furchgott et al., 1961). Photorelaxation in rabbit aorta was shown by Furchgott *et al.*, to peak near 310nm with a shoulder near 350nm. The role of NO_2^- in photorelaxation was implicated when a dominant peak was shown at 355nm following incubation with NO_2^-(Furchgott, 1971). More recently Rodriguez *et al.*, have shown that in rat vascular tissue RSNOs, RNNOs and NO_2^- have photoactivity *in vitro* but NO_3^- shows no appreciable activity. They calculated the action spectra for NO release from RSNOs to be 310-340nm and from NO_2^- 310nm and 350nm(Rodriguez et al., 2003), both these ranges lie within the spectrum of UVA.

It has been hypothesised that NO-related products stored in human skin may play a role in the acute response to UV(Paunel et al., 2005). Paunel *et al.*, demonstrated formation of NO due to photo-decomposition of NO_2^- and RSNOs maximal within 20 minutes of UVA exposure of *ex vivo* full thickness human skin(Paunel et al., 2005). Taking the findings of Paunel *et al.*, a step forward, I have now demonstrated this effect in human skin *in vivo* using cutaneous microdialysis. Dialysate sampling

of the superficial vascular dermis revealed an increase in NO-related products maximal 30 minutes after exposure to a biologically relevant dose of UVA ($30J/cm^2$). Gaseous NO released within the dermis will undergo oxidative decomposition on entering the extra-cellular vascular space forming aqueous NO_2^- . Although both RSNOs and NO_2^- will release NO in the UVA spectrum, the relative concentrations of NO_2^- and RSNOs that I have demonstrated in human skin, suggest that NO_2^- is likely to be the predominant photoactive NO-related products. Evidence suggests that NO may play an anti-apoptotic role in human skin following UVR(Suschek et al., 1999;Suschek et al., 2003b;Weller et al., 2003). It is likely that cutaneous NO-related products offer an immediate enzyme-independent source of NO, allowing protection within 30 minutes of exposure to UVR. As proposed by Paunel *et al.,* this enzyme-independent NO release bridges the time gap following UV exposure before the up-regulation of iNOS and enzyme-dependent NO release is maximal(Paunel et al., 2005) (figure 1.13).

Inter-individual variations in the concentration of NO-related products in human saliva, plasma, sweat, epidermis and superficial vascular dermis were observed in the subjects used for this chapter, and were similar in each of the different biological samples. Gladwin has suggested that the majority of NO_2^- in tissues originates from the exogenous intake of NO_2^- and NO_3^- and not from endogenous sources, this results in great variation in tissue NO_2^- levels. In contrast plasma levels of NO_2^- vary only slightly suggesting the existence of regulatory pathways in blood(Gladwin et al., 2005).

Enzyme dependent NO production occurs in all cell types of human skin by at least one of the three NOS isoenzymes, NO is produced constantly by the endothelium at 4nM/s(Marley et al., 2001). I have confirmed the presence of NO-related products in the dermis and epidermis but I have not isolated the storage site to a particular cell type. On exposure to UVA a significant increase in aqueous NO-related products is detected by cutaneous microdialysis, and this increase is reduced in the presence of a local vasoconstrictor. Both NO_2^- and RSNOs are transported in the bloodstream and

are susceptible to photolysis by UVA. I suspect that much of the observed increase in NO-related products following UVA exposure comes directly from the vasculature but with locally bound stores, also contributing.

Hypertension and ischaemic heart disease (IHD) both correlate with latitude(Fleck, 1989;Rostand, 1997), increasing in incidence with distance from the equator. Much of this is probably due to racial and dietary factors, and a correlation has also recently been shown between UV induced Vitamin D synthesis and reduced IHD(Wang et al., 2008). The data presented in this chapter, shows UVR-induced release of skin bound NO-related products, I suggest that this as an alternative mechanism by which UV exposure may have beneficial cardiovascular effects.

I present data which confirms and quantifies the presence of NO-related products both in human epidermis, superficial vascular dermis and skin surface sweat. These species have the capability of releasing NO, and I have demonstrated such release within 30 minutes of UVA exposure. I believe these findings to be of great significance, in the context of the skin as the largest 'organ' of the human body (10-20dm^3), which thus offers a considerable store. I believe that enzyme-independent NO release is an acute mechanism which serves to prevent UV-induced keratinocyte apoptosis(Paunel et al., 2005). It is known that plasma NO-related products can be influenced by dietary intake of NO_3^- and I have demonstrated a relationship between the concentration of plasma NO-related products and those of the superficial vascular dermis and sweat. I postulate that an individual's dietary consumption of green leafy vegetables may in part influence their cutaneous response to UVR.

CHAPTER 5

NO and UVR-INDUCED APOPTOSIS, P53 ACCUMULATION, DNA DAMAGE and REPAIR

5. CHAPTER 5 - NO and UVR-INDUCED APOPTOSIS, P53 ACCUMULATION, DNA DAMAGE and REPAIR

5.1. Introduction

NO has been shown to have paradoxical effects. Its actions depend on its concentration, its interaction with other free radicals, metal ions, and proteins and on the cell type of the organism. NO diffuses readily through the cytoplasm and plasma membranes over several cell diameter distances. Intracellular NO quickly forms NO-related products: NO_3^-, NO_2^-, RSNOs or $ONOO^-$ (figure 1.1). These NO metabolites may play an important role in the biological effects of NO, in particular its genotoxic effects.

Following the recent publication of a technique allowing detection of NO-related products in tissues and biological fluids(Feelisch et al., 2002), NO-related products consisting primarily of NO_3^-, NO_2^- and RSNOs have been quantified in human epidermis, dermis and sweat on the skin surface(Paunel et al., 2005;Weller et al., 1996) (Ch4.4.6). *Ex-vivo* and *in-vivo* evidence suggests that UVA irradiation induces photolysis of RSNO and NO_2^- stored in human skin with subsequent high-output enzyme-independent NO formation, this reaches a maximum 20-30 minutes after the onset of irradiation(Paunel et al., 2005) (Ch4.4.6, figure 4.9-4.11). Following acute enzyme-independent release of NO from human skin, UVR induces the upegulation of iNOS and enzyme-dependent activity becomes important. The upregulation of iNOS starts 8 to 10 hours after UV irradiation and reaches a maximum at approximately 24 hours(Kuhn et al., 1998;Suschek et al., 2001b). Enzyme dependent NO released from the skin following UV irradiation has been shown to be the major mediator of UV erythema in man(Rhodes et al., 2001).

The pleiotropic nature of NO is exemplified when considering its role in cell apoptosis and DNA damage. NO has been shown to exert cytotoxic effects and initiate the onset of apoptosis in mammalian cells(Kroncke et al., 1997), however it

has also been shown to protect against the detrimental actions of superoxide, hydrogen peroxide and alkyl peroxides(Wink et al., 1996b). NO-induced DNA alterations may lead to p53 post-translational modifications, accumulation and activation(Forrester et al., 1996;Messmer and Brune, 1996;Nakaya et al., 2000), thus contributing to the anti-carcinogenic effects of p53. In addition, NO may modulate tumour DNA repair mechanisms by up-regulating poly (ADP-ribose) polymerase (PARP) and the DNA-dependent protein kinase (DNA-PK)(Xu et al., 2002). However, high levels of NO can mutate the p53 gene allowing for selective clonal expansion of p53-mutant cells, and tumorigenesis(Greenblatt et al., 1994). Recent evidence suggests that NO has an anti-apoptotic role in skin after exposure to UVR. iNOS derived NO has been shown to protect rat endothelial cells *in vitro* against UVA-induced apoptosis(Suschek et al., 1999). Enzyme independent mechanisms are also important as the presence of NO_2^- *in vitro* has been shown to protect endothelial cells from UVA induced apoptosis, in a concentration-dependent manner(Suschek et al., 2003b). An anti-apoptotic role of NO has also been demonstrated in murine keratinocytes *in vivo* and human keratinocytes *in vitro* following UVB exposure(Weller et al., 2003).

The type of DNA damage induced by UVR depends on the wavelength of the photons that hit the cell. UVB exposure (290-320nm) results in direct light absorption by DNA, this induces dimerisation reactions between adjacent pyrimidine bases and the formation of DNA PPs (Ch1.6.2.4). DNA PPs are characterised by C to T or CC to TT transitions. The transitions are regarded as 'signature' mutations, only known to be caused by UV(Brash et al., 1991). CPDs in general and thymine dimers in particular, are the major DNA PPs. The remainder of PPs are accounted for by 6-4PPs. 6-4 PPs may be converted into their Dewar isomers upon exposure to near UVR. All UVR wavelengths have been shown to induce DNA PPs(Douki et al., 2003;Kobayashi et al., 2001;Mouret et al., 2006;Sheehan et al., 2002). UVB only contributes to 5% of the UVR reaching the earths surface, the remainder being composed of UVA (320-400nm). However, UVB induces the majority of DNA damage due to the strong absorption of DNA at UVB wavelengths(Douki et al.,

2003). CPD formation in skin has been shown to peak at wavelengths near 300nm(Chadwick et al., 1995;Freeman et al., 1989), although mean levels following narrow band (NB) UVB exposure (311nm) were found to be comparable to those induced by broad band (BB) UVB (295-320nm)(Snellman et al., 2003).

Critical proteins involved in the response to DNA damage include the p53 tumour suppressor protein. p53 is an important transcriptional factor and also directly reacts with proteins such as NER-associated regulatory proteins. Upon activation of p53, cells have two possible outcomes(Chow and Tron, 2005), cell cycle arrest with:

- DNA repair
- Apoptosis

Evidence for the involvement of PPs in skin carcinogenesis is provided by the finding of C to T and CC to TT tandem mutations in p53 tumour suppressor genes isolated from skin tumours(Brash et al., 1991;Brash et al., 1996;Dumaz et al., 1993;Ziegler et al., 1993). Mutated p53 is unable to induce apoptosis, therefore allowing for the persistence of DNA damaged cells and mutagenesis. The lack of repair of PPs in XP further indicates an association between DNA photodamage and skin cancer (Dumaz et al., 1993;Nakazawa et al., 1994;Ziegler et al., 1993). In XP patients the incidence of NMSC is more than 1000 times greater than in the normal population.

In the setting of NO research, translational clinical research is of paramount importance if we are to accurately determine the role of this pleiotropic free radical in the processes of: UVR-induced apoptosis, p53 accumulation and DNA damage/repair in human skin under normal physiological conditions.

5.2. Aims

The aim of this study was:

- To determine the influence of physiologically relevant concentrations of UVR on human skin *in vivo*, in the presence/absence of exogenous NO, on:
    - Apoptosis
    - p53 accumulation
    - DNA damage and repair

5.3. Methods

All methods common to more than one study are detailed in Chapter 2.

5.3.1. Study volunteers

Two individual studies were performed, each using healthy volunteers.

- To determine the effects of exogenous NO on human keratinocyte apoptosis and p53 accumulation post UVB – seven subjects, age range 22-44 years, 3 male.
- To determine the effects of exogenous NO on human keratinocyte DNA damage/repair post UVB – ten subjects, age range 18-35 years, 5 male.

5.3.2. UV source

The UVB source used was a Waldmann UV 801 BL unit (Waldmann, Villingen-Schwenningen, Germany) which contains a bank of ten TL 20W/01RS fluorescent lamps (Philips, Eindhovan, Holland). TL 20W/01RS lamps emit a narrow peak around 311nm exclusively. Using this apparatus 1SED of UVR is delivered over 30 seconds to a $10cm^2$ area (10mJ/0.33mW) of skin at a distance of 10cm from the irradiating bulbs.

5.3.3. Preparation and application of topical NO donors

For both experiments Ze-NO (33% wt/wt) was used as an NO donor. This is an inert topical NO donor which we have considerable experience using(Mowbray et al., 2008). At the time of each application 100mg of Ze-NO powder was mixed with 200mg aqueous cream BP® and applied immediately to a 4cm^2 area on the volar aspect of the forearm (figure 5.1).

5.3.4. Apoptosis study design

Day 1 - graded doses of UVB to volar forearm for determination of MED.

Day 2 - two hours prior to attending the department subjects applied aqueous cream BP® to one 1 x 4cm^2 area (area 1) and Ze-NO to a second 1 x 4cm^2 area (area 2), both on the volar aspect of the non-dominant forearm. After reading of the subjects MED, topical applications were removed and each area cleaned with an alcohol wipe (Alcotip swab, Universal Hospital supplies Ltd, UK). An area measuring 1 x 2cm^2 was irradiated with 2MED UVB at the distal end of each of areas 1 and 2 (see figure 5.1 for an example of the layout). Aqueous cream BP® and Ze-NO were reapplied immediately to areas 1 and 2 respectively, subjects continued to apply the topical formulations eight hourly until their final visit 24 hours later.

Day 3 - twenty four hours following UVB irradiation topical applications were removed as described previously. One 4mm full thickness skin punch biopsy was taken from an irradiated and unirradiated site within each of areas 1 and 2 (total of four biopsies).

5.3.5. DNA damage study design

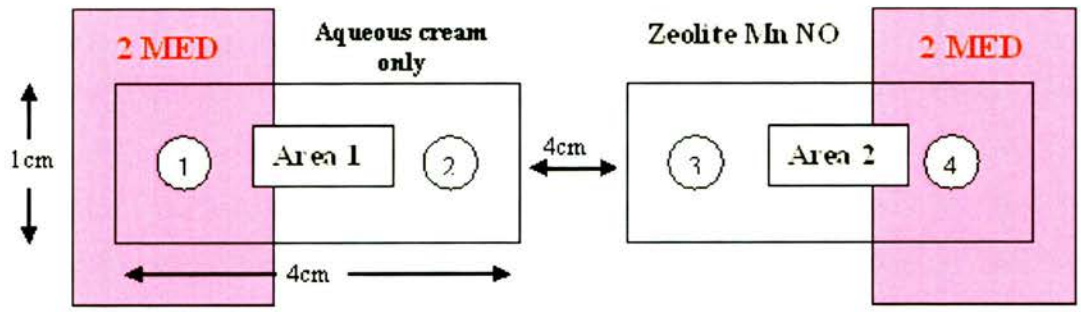
Day 1 - graded doses of UVB to volar forearm for determination of MED.

Day 2 - two hours prior to attending the department subjects applied aqueous cream BP® to area 1 and Ze-NO to area 2 on the volar aspect of the non-dominant forearm (figure 5.1). Twenty four hours after being exposed to graded doses of UVB subjects attended to have their MED read. Subjects with the same MED were selected so as to

minimise inter-individual variation in DNA repair. Topical applications were removed as described previously. Areas 1 and 2 were irradiated with 2MED UVB, aqueous cream BP® or Ze-NO were reapplied to areas 1 and 2 respectively, immediately after irradiation. Subjects continued to apply aqueous cream BP® to area 1 and Ze-NO to area 2 eight hourly until their final visit on day 3.

Day 3 - two hours prior to their day 3 visit subjects applied aqueous cream BP® to area 3 and Ze-NO to area 4 on the volar aspect of the dominant forearm. At their final visit on day 3 (30 hours following UVB irradiation), topical applications were removed as described previously. Areas 3 and 4 were irradiated with 2MED UVB. Following irradiation one 4mm full thickness skin punch biopsy was taken immediately from each of the four sites 5-8, thereafter one 4mm punch biopsy was taken from each of the four sites 1-4 (total of eight biopsies).

30 hour panel, non-dominant volar forearm (4 applications)



15 minute panel, dominant volar forearm (1 application)

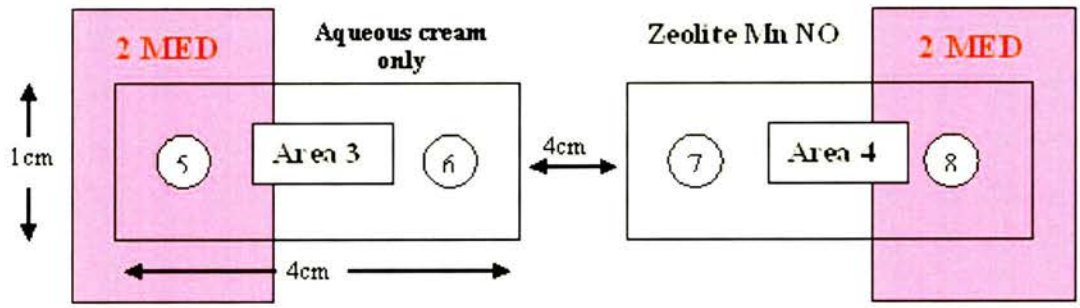


Figure 5.1 *Diagrammatic representation of sites of topical applications, irradiation and punch biopsies for DNA damage experiment*

The 30 hour panel represents a similar layout to that used in the apoptosis, p53 study. The fifteen minute panel represents maximal DNA damage, approximately 50% repair has taken place by 30 hours.

5.3.6. Histology & Immunohistochemical staining

All 4mm full thickness skin punch biopsies were fixed in 4% buffered formaldehyde and then processed for histology. Tissues were embedded in paraffin and 4µm sections were cut. Two sections from each biopsy were stained with H&E, the remainder were reserved for immunohistochemical (IHC) studies. A Dako EnVision+ System-HRP (DAB) K4007 was used for IHC staining (DakoCytomation Carpintera, California). Initially paraffin sections were de-waxed and rehydrated, following which any endogenous peroxidase activity was quenched by incubating the specimen with peroxidase block. The specimen was then incubated with primary antibody, followed by incubation with the labelled polymer. Staining was then

completed by incubation with DAB+ substrate-chromogen, which results in brown coloured precipitate at the antigen site. Finally, slides were counterstained in haematoxylin before dehydration and cover slipping. The antibodies used for IHC staining included:

- Anti-ACTIVE® Caspase-3 pAb (Promega corporation, Madison, USA) - recognises the cleaved active form of caspase-3.
- Monoclonal Mouse Anti-Human p53 Protein (DO-7 DakoCytomation, Glostrup, Denmark) - labels both wild-type and mutant p53.
- Anti-Thymine Dimer, clone KTM53 (Kamiya BiomedicalCompany, Seattle, USA), which stains CPD positive keratinocytes by reacting specifically with thymine dimers produced by UV irradiation in double- or single-stranded DNA.

Positive and negative control slides were included in all tests.

5.3.7. Morphological detection of apoptosis

One H&E section from each subject's 4 sites of the apoptosis study was quantified for sunburn/apoptotic keratinocytes. Sunburn cells were identified as epidermal cells with shrunken eosinophilic cytoplasm and a condensed nucleus (figure 5.5c,d)(Ch1.6.2.3).

5.3.8. Quantification of positive cells (H&E and IHC)

For analysis of the staining results and preparation of images a multi-colour microscope (Leitz), Q imaging monochrome camera and Q Capture Pro computer software (Media Cybernetics, UK) were used. Positive and negative cells were counted at high magnification using a x40 objective lens. Following IHC staining cells were classed as positive if they showed uniform dark brown nuclear staining (figure 5.5g,h,k,l,o,p,s,t). All cells were separately counted with the aid of Photoshop 5.5 and a grid (Adobe corporation, San Jose, California), the Photoshop 'pencil tool' was used to mark each cell as it was manually counted. In this way a permanent record of the counted cells was retained. Counting was performed by three blinded

observers for the apoptosis study and two blinded observers for the DNA damage study. The mean number and percentage of positive cells per high power field was calculated for each section.

5.4. Results

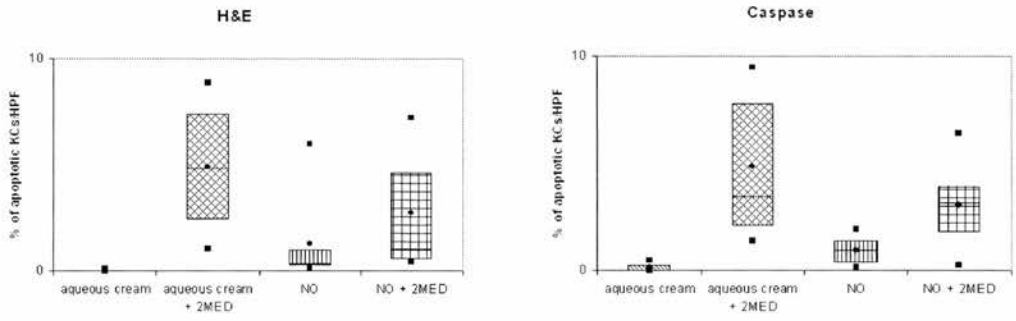
5.4.1. Exogenous NO causes a moderate reduction in apoptosis 24 hours after UVB exposure compared with vehicle control

To study the effect of physiologically relevant concentrations of exogenous NO on UVB-induced keratinocyte apoptosis, Ze-NO donor was applied two hours before and eight hourly after exposure to 2MED UVB. Apoptosis was quantified using two methods:

- Morphological changes on H&E sections (sunburn cells).
- Presence of activated Caspase 3.

Both methods of apoptosis quantification suggested that exogenous NO-induces a moderate reduction in keratinocyte apoptosis when assessed 24 hours following UVB exposure (figure 5.2 a & b, figure 5.4).

- H&E (% positive apoptotic keratinocytes/HPF) – aqueous cream only 0.04 ± 0.03, aqueous cream + 2MED 4.92 ± 3.06, NO only 1.28 ± 2.13, NO + 2MED 2.72 ± 2.91.
- Activated caspase 3 (% positive apoptotic keratinocytes/HPF) - aqueous cream only 0.13 ± 0.19, aqueous cream + 2MED 4.87 ± 3.46, NO only 0.92 ± 0.66, NO + 2MED 3.06 ± 2.



a) H&E morphological changes *b) activated caspase 3 positive keratinocytes*

Figure 5.2 *Percentage apoptotic keratinocytes per high power field of human epidermis 24 hours post exposure to 2MED UVB, in the presence or absence of exogenous NO a) H&E morphological changes, b) activated caspase 3 positive keratinocytes*

Boxplots represent median and interquartile range, ▪ maximum and minimum values, • mean, n=7.

5.4.2. A reduction in stable p53 is observed in the presence of exogenous NO 24 hours post 2MED UVB exposure compared with vehicle control

In addition to investigating the effects of exogenous NO on UVB-induced keratinocyte apoptosis, I was also interested in the effects of NO on the accumulation of stable p53. Biopsies taken 24 hours after 2 MED UVB, in the presence or absence of Ze-NO donor, were stained for the presence of stable p53. The changes in p53 accumulation mirrored those of keratinocyte apoptosis. If exogenous NO was present for two hours before and for 24 hours after 2MED UVB, a reduction in the accumulation of stable p53 was observed (figure 5.3 & 5.4). This would suggest that exogenous NO has an anti-apoptotic role following UVB exposure which is related to a reduction in the accumulation of stable p53. A decrease in stable p53 will lead to a reduction in cell cycle arrest and consequently apoptosis.

- P53 (% p53 positive keratinocytes/HPF) – aqueous cream only 0.01 ± 0.02, aqueous cream + 2MED 11.68 ± 6.82, NO only 3.61 ± 6.38, NO + 2MED 6.86 ± 6.28.

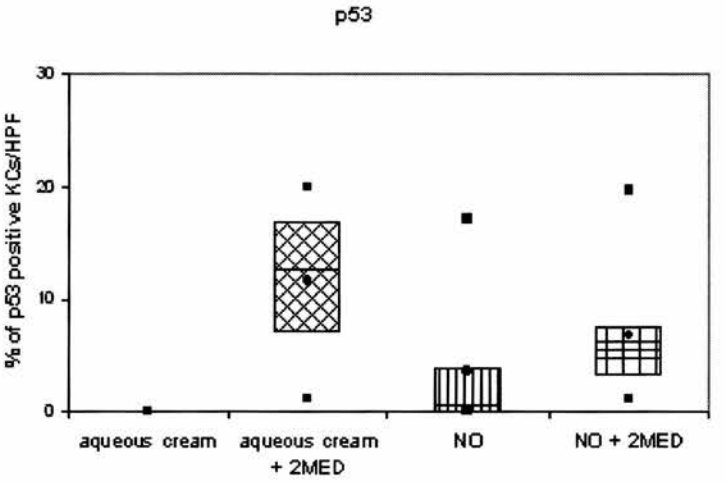


Figure 5.3 *Percentage of p53 positive keratinocytes per high power field of human epidermis 24 hours post exposure to 2MED UVB, in the presence or absence of exogenous NO*

Boxplots represent median and interquartile range, ▪ maximum and minimum values, • mean, n=7.

Figure 5.4 *H & E and immunohistochemical staining of skin following topical application of Ze-NO and control in the presence or absence of UVR, apoptosis and DNA damage/repair studies (x40)*

c reveals 'sunburn cells' with shrunken eosinophilic cytoplasm and a condensed nucleus. g & h reveal caspase positive cells in brown. k & l reveal p53 positive cells in brown. o,p,s and t reveal CPD positive cells in brown.

5.4.3. No significant difference was found between data from three independent blinded observers

In the apoptosis/p53 study positive and negative keratinocytes in all sections from all four sites of each subject were counted by three individual blinded observers. When using such IHC techniques variability in the staining of different cells, in addition to variability in interpretation of results by different observers, is well documented. Despite the opportunities for methodological variation, ANOVA revealed no significant difference between the results of each individual observer (figure 5.5).

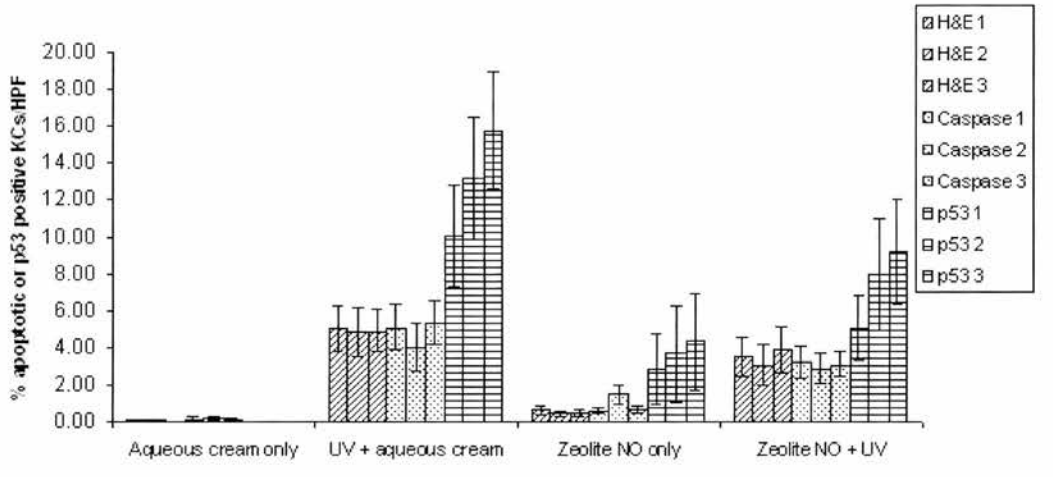


Figure 5.5*Comparison of data from three independent blinded observers for quantification of apoptotic keratinocytes on H&E morphology, activated caspase 3 and p53 positive cells*

Mean % positive cells/high power field ± SEM of 7 subjects are shown.

5.4.4. No difference was observed between exogenous NO or vehicle in CPD positive epidermal keratinocytes 30 hours post 2MED UVB

In view of the reduction in keratinocyte apoptosis observed in the presence of exogenous NO, I wished to explore whether NO influences DNA damage or repair processes following UVB. Ze-NO donor and vehicle control were applied to human skin two hours before, and eight hourly for 30 hours after, 2MED UVB. Biopsies

were taken from an irradiated and unirradiated site for both Ze-NO donor and vehicle control at 15 minutes (maximal DNA damage) and 30 hours (50% DNA repair) following UVB (figure 5.1). No significant difference in DNA damage was observed between Ze-NO donor and vehicle control at either 15 minutes or 30 hours following UVB (figure 5.4 & 5.6).

- 15 minutes (% KTM53 positive keratinocytes/HPF) – aqueous cream + 2MED 83.44 ± 10.78, NO + 2MED 76.11 ± 15.79
- 30 hours (% KTM53 positive keratinocytes/HPF) - aqueous cream + 2MED 71.05 ± 13.91, NO + 2MED 62.27 ± 23.76

It is interesting to observe that 30 hours following UVB, a time by which one would expect 50% of DNA repair to have taken place, greater inter-individual variation in the number of keratinocytes expressing DNA damage was seen in skin treated with Ze-NO than that treated with vehicle control. This data would suggest that NO may be affecting the kinetics DNA repair processes in some individuals following exposure to UVB.

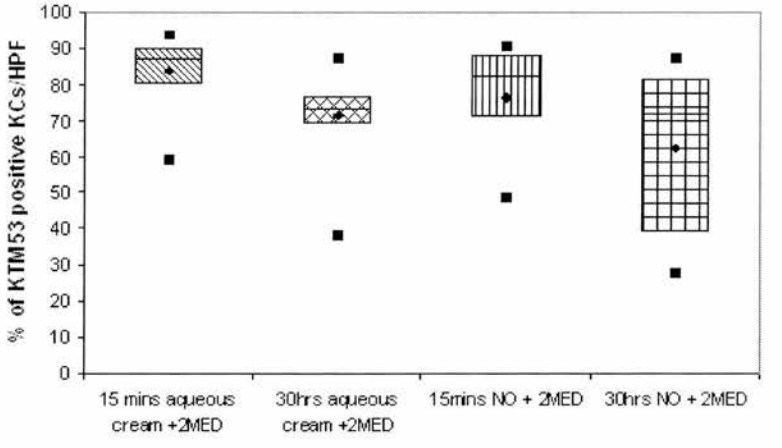


Figure 5.6 *Percentage of CPD positive epidermal keratinocytes per high power field 15 minutes and 30 hours post exposure to 2MED UVB, in the presence and absence of exogenous NO*

Boxplots represent median and interquartile range, ▪ maximum and minimum values, • mean, n=10.

5.4.5. Exogenous NO donor alone (unirradiated) causes no apoptosis or DNA damage

It is important to note that neither Ze-NO or aqueous cream resulted in any DNA damage in unirradiated skin (figure 5.7).

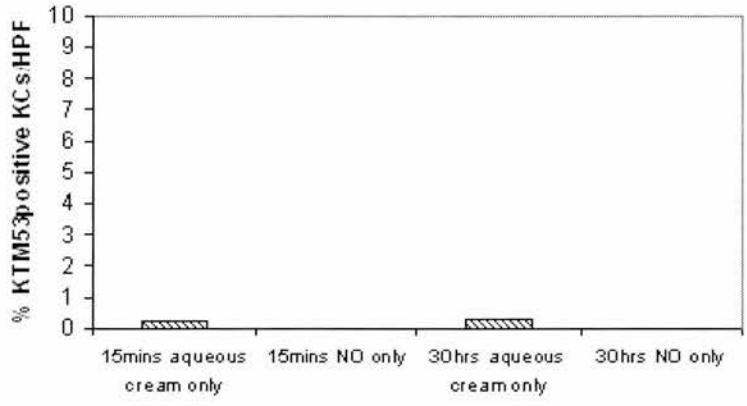


Figure 5.7 *Percentage of CPD positive epidermal keratinocytes per high power field 15 minutes and 30 hours post application of exogenous NO and control, unirradiated (n=10)*

5.4.6. Inter-individual variation in DNA repair is observed at 30 hours post 2MED UVB, despite controlling for skin type and MED

Maximum DNA damage in the form of CPDs is observed within 15 minutes of exposure to UVB. Thirty hours following UVB exposure one would expect 50% of CPD repair to have taken place in epidermal keratinocytes(Young et al., 1996). Skin biopsies were taken both 15 minutes and 24 hours following 2MED UVB in the presence and absence of a Ze-NO donor. A percentage change in the percentage of CPD positive keratinocytes per high power field was calculated as a measure of effective CPD repair 30 hours following UVB. No significant difference in the mean number of CPD positive keratinocytes was observed 30 hours following UVB between Ze-NO (11.53 ± 43.31) and vehicle control (13.49 ± 20.05) treated sites, however the presence of Ze-NO induced a wide inter-individual variation in the amount of CPD repair (figure 5.8).

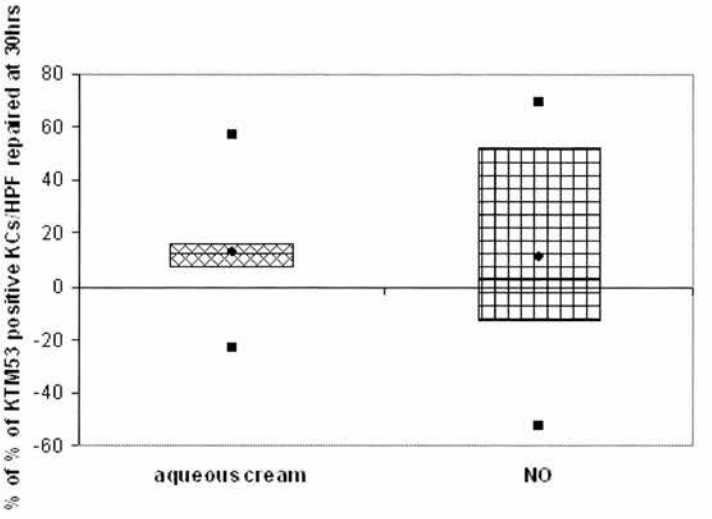


Figure 5.8 *Percentage of percentage CPD positive epidermal keratinocytes per high power field repaired 30 hours post exposure to 2MED UVB, in the presence or absence of exogenous NO*

Boxplots represent median and interquartile range, ▪ maximum and minimum values, • mean, n=10.

5.5. Discussion

The experiments performed in this chapter represent the first *in vivo* study designed to determine the role of physiological concentrations of exogenous NO on keratinocytes in human skin *in vivo* following UVB exposure. The major outcome measures were quantification of epidermal keratinocyte:

- Apoptosis
- Accumulation of stable p53
- DNA damage/repair in the form of CPDs

A concentration and application frequency of inert stable NO donor(Mowbray et al., 2008) was used which induced comparable erythema to that observed in human skin 24 hours after 0.75MED UVB (figure 6.3). The importance in pursuing translational *in vivo* clinical research when studying the biological role of NO is enormous in view of the pleiotropic nature of this free radical. All investigations of NO need to be performed in situations which are as close to the normal *in vivo* biological set-up as possible, in order to formulate solid hypotheses as to the actual role of this 'double

edged sword' *in vivo*. In addition to adding weight to hypotheses regarding the role of NO following UVR exposure in normal human skin, the data that is presented in this chapter is also useful for those studying the potential uses of exogenous NO or its metabolites as a topical treatment. NO donors are already in use clinically in various forms such as: glyceryl trinitrate (GTN) in relief of acute angina, GTN ointments for anal fissures and transdermal patches in heart failure and chronic angina. Potential future uses of topical NO-related products include: antimicrobial(Weller et al., 1998), vasodilatory in Raynaud's disease(Tucker et al., 1999), wound healing particularly in diabetics(Weller and Finnen, 2006a), and antithrombotic as a coating for stents, extracorporeal circuits and catheters used in interventional cardiological procedures, surgery and renal replacement therapy and catheters(Miller and Megson, 2007a).

The experiments presented in this chapter demonstrate that physiological concentrations of exogenous NO protect against UVB-induced epidermal keratinocyte apoptosis 24 hours following exposure to 2MED (figure 5.2 a & b). This observation was made using two different objective methods of apoptosis quantification, suitable for use in paraffin embedded skin sections, both are recognised as reproducible methods in comparison with other limited options(Baima and Sticherling, 2002). Apoptosis is a continuous process and not always synchronous, each method of quantification assesses a different stage in this process and in some instances cells indicated by particular assays may not be the same. For example, the change in nuclear morphology often does not coincide with the appearance of detectable DNA strand breaks in cells(Willingham, 1999). The most specific assay, and one which was chosen for use in these experiments, is perhaps the oldest, the detection of nuclear morphological shape changes, and in the case of keratinocytes the presence of 'sunburn cells'(DANIELS, Jr. et al., 1961). This assay has advantages when used in the context of *in vivo* research where many uncontrollable biological variables exist, it is an assay which shows great reproducibility between observers, as was demonstrated in this study (figure 5.5). The second method for quantification of apoptosis which was employed is the

quantification of active caspase-3. Cysteine proteases called caspases are the mediators of the execution phase of apoptosis, caspase-3 is regarded as a central player in this process and is the most widely studied caspase.

Mirroring the effects of NO on apoptosis, exogenous NO induced a reduction in the accumulation of stable p53 in epidermal keratinocytes 24 hours after exposure to 2MED UVB, when compared with vehicle control (figure 5.3). Although again not reaching significance, I regard these results as being informative in guiding my hypotheses. Assays which employ IHC staining, such as the one used in this experiment, have both advantages and disadvantages. Advantages include: cost, labour and preservation of morphological function. Disadvantages include: problems related to sensitivity of the antibody used, dilutions, fixation artefacts, differences in methods of evaluation and inter-observer variability(Chadwick et al., 1995;Kay et al., 1996). The many factors leading to the potential for variability with IHC are difficult to fully control for, in addition when performing *in vivo* invasive procedures in man subject numbers tend to be limited. This recognised potential of variability increases the importance of the observation that the results from three independent blinded observers were reproducible (figure 5.5). Published literature supports this finding, data suggests that variance within an institution is small, where criteria are likely to be agreed by all observers. Difficulties arise when one tries to make comparisons of p53 immunohistochemistry between institutions(Kay et al., 1996).

In the first phase of the experiments presented in this chapter 'apoptosis study', a moderate reduction was observed in both keratinocyte apoptosis and stable p53 accumulation if exogenous NO is present before and for 24 hours after UVB exposure. This reduction was not found to be significant when compared to the control situation. Despite this lack of significance I believe these results are important in guiding our hypotheses regarding the biological actions of NO. When interpreting these results one must not forget the difficulties in performing invasive *in vivo* clinical studies with the obvious limitations on subject numbers, in addition to the complexities of individual biological variation. Of note the absence of a

significant increase in keratinocyte apoptosis and p53 accumulation, in samples exposed to exogenous NO both in the presence and absence of UVR exposure, is an important finding if exogenous NO is to be considered as a potential therapeutic topical application.

The data presented from the 'apoptosis study' leads me to hypothesise that NO is acting anti-apoptotically in human skin post UVB exposure, I would suggest that a reduction in the accumulation of stable p53 in the presence of exogenous NO is playing a role in this process. p53 has numerous vital cellular functions: gene transcription, DNA repair, cell cycle control, genomic stability and apoptosis. In contrast to the data presented in this chapter, high NO concentrations have been found to induce WT p53 accumulation(Forrester et al., 1996;Ho et al., 1996), once again this highlights the importance of using physiologically relevant concentrations of NO which in turn should lead to the most biologically relevant hypotheses. The process of DNA damage induces the accumulation of p53, a sufficient accumulation of normal p53 will result in a detectable increase in p53 seen on IHC staining. The picture is a little more complicated however, as mutant p53 is more stable than WT and can also stain positively. Additional reasons for positive p53 include functional or conformational abnormality in the protein not detectable at the gene level, and the binding of the protein product to other molecules(Kay et al., 1996). The data presented in this chapter demonstrates a corresponding reduction in apoptosis and p53 in the presence of exogenous NO, hence I would suggest that the majority of the reduction in stable p53 represents a reduction in the accumulation of active p53.

The reduction in apoptosis and p53 seen in the first phase of experiments 'apoptosis study' led me to question the role of NO in DNA damage/repair. Is NO resulting in:

- A reduction in the number of epidermal keratinocytes suffering from significant DNA damage?
- An increase in DNA repair within epidermal keratinocytes?

Either scenario would result in less p53 accumulation and therefore less apoptosis. Alternatively, is NO having more deleterious consequences by inhibiting the important regulatory process of p53 up-regulation and apoptosis, thus allowing the persistence of DNA damaged cells with an increased risk of mutagenesis? I endeavoured to answer these important questions by performing a second phase of experiments, designed to examine the role of exogenous NO in DNA damage/repair processes following UVB exposure. No significant difference in maximal DNA damage was observed between keratinocytes exposed to UVB in the presence of exogenous NO or control (figure 5.6).

As has been the experience of many other researchers, the assessment of DNA damage in keratinocytes is not without its difficulties, despite it being a subject which has received widespread attention for many years. Firstly, the actual rate of repair of UV induced DNA damage is widely debated and published figures for a $t_{1/2}$ of repair of CPD PPs ranges from 40-50% removal within 1 hour(D'Ambrosio et al., 1981;Reusch et al., 1988) to a $t_{1/2}$ of 33.3 hours(Young et al., 1996), with one group suggesting that almost complete removal of CPDs can take up to 72 hours after exposure to as little as 1.2MED(de Winter et al., 2001). Some of these variations can be explained by differing methods of CPD quantification and experimental protocols. 33.3hrs is the CPD $t_{1/2}$ demonstrated by the first study to use subjects with well defined skin types I and II, directly comparable to the skin types of our subjects, this study also used a reasonable sample size. These observations prompted me to choose 30 hours as the time point for measuring DNA repair in the experiments presented in this chapter(Young et al., 1996).

Both UVA and UVB have been implicated in the pathophysiology of NO in the response of human skin to UVR. UVB induces the majority of DNA damage with CPD formation in human skin peaking at wavelengths near 300nm(Chadwick et al., 1995;Freeman et al., 1989). Snellman *et al.*, found mean levels of CPDs following NB UVB exposure (311nm) to be comparable to those induced by BB UVB (295-315nm)(Snellman et al., 2003). UVR has been used to treat various skin diseases for

many years. There has been a recent increase in the use of NB UVB to treat: psoriasis, vitiligo and atopic dermatitis, this follows evidence suggesting that it is more therapeutic than BB UVB(Coven et al., 1997;Walters et al., 1999). Interestingly Kunisada *et al.*, have recently published work suggesting NB UVB induces more carcinogenesis than BB UVB(Kunisada et al., 2007). Taking all these factors into account, coupled with the established *in vivo* murine data(Weller et al., 2003), and the practicality of use of our departmental UVR sources for the proposed experimental protocol, a NB UVB source was used for the studies of both apoptosis and DNA damage/repair.

Interestingly, minimal inter-individual variation was observed in the maximum percentage of CPD positive cells (15 minutes post UVR), in the presence of either NO donor or vehicle control (figure 5.6). In contrast, although no significant difference was observed in the reduction of CPD positive keratinocytes at 30 hours post exposure (50% repair), skin exposed to UVB in the presence of NO demonstrates much greater inter-individual variability in CPD positive keratinocytes at 30 hours than at 15 minutes (figures 5.6 & 5.8). This observed inter-individual variability is not seen in control samples (figure 5.6 & 5.8). Inter-individual variability in DNA repair kinetics is a widely recognised phenomenon(Bruze et al., 1989;Bykov et al., 1999;Sheehan et al., 2002;Young et al., 1996). Bruze *et al.*, found that there was significant inter-individual variation in thymine dimer yield but that this was not related to erythemal response(Bruze et al., 1989). Similarly Young *et al.*, revealed a wide variation in CPD repair following solar simulated radiation exposure, this variation was not related to differing skin types(Young et al., 1996). Bykov *et al.*, report findings similar to those of our study, they observed an inter-individual variation in DNA damage after 2MED UVR of six-fold, this variation increases to 20 fold when DNA repair is considered(Bykov et al., 1999). There remains no strong data that suggests a relationship between skin type or erythemal response and CPD accumulation. Some insight has however been provided by Sheehan *et al.*, who used single exposure data (0.65 and 2 MED) to demonstrate that CPD accumulation is related to the physical dose (J/cm^2) of UVR and is independent

of skin type(Sheehan et al., 2002). When investigating DNA repair kinetics, Bruze *et al.* made the interesting observation that repair rates of CPDs *in vivo* in human skin are faster than those of cultured human keratinocytes or fibroblasts *in vitro*(Bruze et al., 1989). This observation reinforces the importance of translational clinical research in human subjects when one is studying the effects of NO in DNA damage/repair.

Aware of the wide variation in an individual's ability to repair DNA damaged keratinocytes I attempted to minimise this problem by selecting for skin type I and II subjects only, and thereafter selecting for subjects with the same MED. Consequently, of our ten subjects eight had the same MED of 6.9 SED, two subjects had an MED of 8.7 SED. SED is a standard measure of UVR dose, one SED is equivalent to an erythemal effective radiant exposure of 100J m^{-2} (Diffey, 2002)(Ch1.6.2.2). An additional source of potential variation lies in the IHC assay itself, maximal CPD accumulation 15 minutes following UVR exposure manifests as very strongly positive and uniform staining in all affected keratinocytes (figure 5.4 o&p). In contrast, 30 hours after UVR exposure, when approximately 50% of CPD repair has taken place, one would envisage keratinocytes at varying stages of CPD repair. This period of 'mid-repair' is manifest on IHC staining as keratinocytes which exhibit variable degrees of staining (figure 5.4 s&t), thus making accurate quantification more variable, both in NO and control treated specimens. Despite the potential methodological variation discussed, the degree of variation in DNA repair between individuals which was observed in the presence of exogenous NO, would suggest that NO may affect the DNA repair kinetics in some subjects. Exploration of such a hypothesis would require further experiments with greater subject numbers.

Some of the inter-individual variation in DNA repair may be explained by an individuals NO stores, already present as NO-related products stored in the skin and in sweat on the skin surface. Both skin and sweat nitrososepices are sources of UVR-induced enzyme-independent NO production, and both are influenced by an individuals plasma concentration of NO-related products (Ch4.4.6). Plasma

concentration of NO-related products is itself dependent on an individuals dietary NO_3^- ingestion(Lundberg and Govoni, 2004). One option of further controlling for inter-individual variation would be to introduce a NO_3^- controlled diet to each subject prior to commencing the apoptosis and DNA damage/repair experiments, this would improve control of inter-individual variation in skin stores of NO-related products.

An additional factor which may serve to minimise inter-individual variation may be provided by tighter determination of an individuals MED, such that even closer matching of MEDs can be achieved. Damian *et al.*, suggest calculating the melanin index of each subject using a reflectance melanin meter, following this one can achieve tighter determination of the MED around narrower increments, with test doses clustered around the MED(Damian et al., 1997). Having determined each individuals MED, I would still advocate selection of subjects with the same MED, thus ensuring equal biological and physical doses of UVR are administered, and hopefully minimising inter-individual variability.

With a variety of factors potentially influencing inter-individual variability in DNA repair following UVR exposure, it is difficult to make solid hypotheses regarding the role of NO in this process. As indicated previously, such invasive experiments performed in human subjects always result in small subject numbers who each bring with them their own personal biological variability. This should not detract from the importance of such experiments and the guidance that they offer in directing future research. Importantly, in the case of exogenous NO, no significant increase in DNA damage was seen on its topical application in the presence or absence of UVR. Further work in this area needs to be directed towards the potential subtle but important effects of differing concentrations of NO on apoptosis and DNA damage/repair following UVR. Such experiments should include NO both in the form of endogenous stores of NO-related products, or exogenous NO as used in these experiments. As has been highlighted throughout this discussion, it is essential that this work is directed towards *in vivo* clinical studies in order to fully elucidate the role of NO in the normal biological situation.

CHAPTER 6

NO and UVR-INDUCED UPREGULATION OF ARGINASE IN HUMAN SKIN

6. CHAPTER 6 - NO and UVR-INDUCED UPREGULATION OF ARGINASE IN HUMAN SKIN

6.1. Introduction

The skin is the largest organ in the human body and is a primary target for many environmental stimuli in particular UVR. As has been demonstrated in the previous chapters, much evidence now exists to suggest that NO plays a major role in UVR-induced cutaneous effects. All three NOS enzymes have been shown to exist in human skin in varying amounts. Enzyme-dependent NO release following UVR is secondary to the upregulation of iNOS, which starts eight to ten hours after UV exposure and reaches a maximum at approximately 24 hours(Kuhn et al., 1998;Suschek et al., 2001b). Enzyme-dependent NO is the major mediator of UV erythema in man(Rhodes et al., 2001). NO can also be released by enzyme-independent mechanisms, UVR induces the photolysis of NO-related products stored in human skin, this release occurs within 20-30 minutes of UVR exposure(Paunel et al., 2005) (Ch 4.4.6).

NO is a free radical which is well known for its pleiotropic actions, the actions of NO are determined by the concentration of NO, the target cell and the microenvironment. The biphasic nature of NO is exemplified by the role it plays in both inflammation (Ch1.4.2) and apoptosis (Ch1.6.3.4). In the experiments described in the preceding chapters the NO environment has been manipulated by the use of an inert topical NO donor (Ze-NO), all experiments were performed using physiologically relevant concentrations of NO, in attempt to best mimic the normal biological situation. Varying the concentration of NO in human skin has the potential to cause variable effects on biological processes, such as apoptosis and inflammation. In view of the dramatic effects that differing NO concentrations can have on human skin, it is likely that the human body has an in-built mechanism for the control of NO generation and concentration in human skin.

NO is synthesised from L-arginine by all three NOS isoenzymes, appropriate iNOS activity following UVR-induced iNOS upregulation depends on the availability of L-arginine, in addition to expression and activity of CATs, which allow L-arginine transport into cells (Ch1.3.2). It has been shown that in cytokine activated endothelial cells, L-arginine supply at physiological levels can restrict eNOS activity(Suschek et al., 2003a). Evidence exists of a tight link between iNOS and the urea cycle, arginase competes for L-arginine as a substrate which it hydrolyses to ornithine and urea as part of the urea cycle (figure 6.8). Arginase has been shown to be effective in down regulating NO production(Mori and Gotoh, 2000).

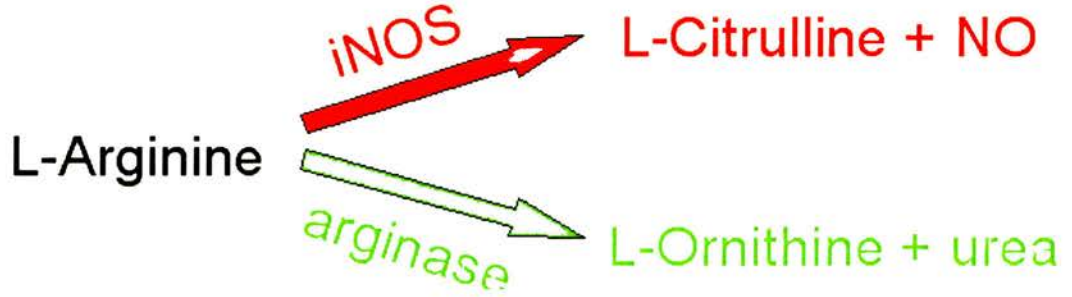


Figure 6.1 *L-Arginine is a common substrate for both iNOS and arginase*

6.2. Aims

The aim of this study was:

- To further investigate preliminary findings which suggest a feedback control mechanism between iNOS and arginase, competing for the common substrate L-Arginine.

6.3. Methods

All methods common to more than one study are detailed in Chapter 2.

6.3.1. Study volunteers

Eight healthy volunteers participated in the study, age range 21-50 years, 1 male.

6.3.2. UV source

The UVB source used was a Waldmann UV 801 BL unit (Waldmann, Villingen-Schwenningen, Germany) which contains a bank of ten TL 20W/01RS fluorescent lamps (Philips, Eindhovan, Holland). TL 20W/01RS lamps emit a narrow peak around 311nm exclusively. Using this apparatus 1SED of UVR is delivered over 30 seconds to a $10cm^2$ area (10mJ/0.33mW) of skin at a distance of 10cm from the irradiating bulbs (figure 6.4).

6.3.3. Preparation and application of topical NO donors

Ze-NO (33% wt/wt) was used as an NO donor. This is an inert topical NO donor which I have considerable experience using(Mowbray et al., 2008). At the time of each application Ze-NO powder was mixed with aqueous cream BP® and applied immediately to an area on the lower back (figure 6.2). Ze-NO was reapplied immediately after UVR on day 2 and left in place for 24 hours.

6.3.4. Study protocol

Each volunteer attended the department a total of five times covering four separate days:

Day 1 - graded doses of UVB to volar forearm for determination of MED.

Day 2, 0 hours - reading of MED, irradiation of lower back and Ze-NO application.

Day 2, 1 hour - 20 tape strips from each of 5 sites on the lower back.

Day 3 - 20 tape strips from each of 5 sites on the lower back.

Day 14 - 20 tape strips from each of 5 sites on the lower back.

Five 9cm by 3cm strips each 1cm apart were marked on the lower back (figure 6.2 & 6.3).

- Strip 1 – unirradiated
- Strip 2 - 0.75MED
- Strip 3 - 1 MED
- Strip 4 - 2MED
- Strip 5 - 33% Ze-NO

Ze-NO was removed with distilled water followed by an alcohol swab (Alcotip, UHS Ltd, UK) prior to tape stripping.

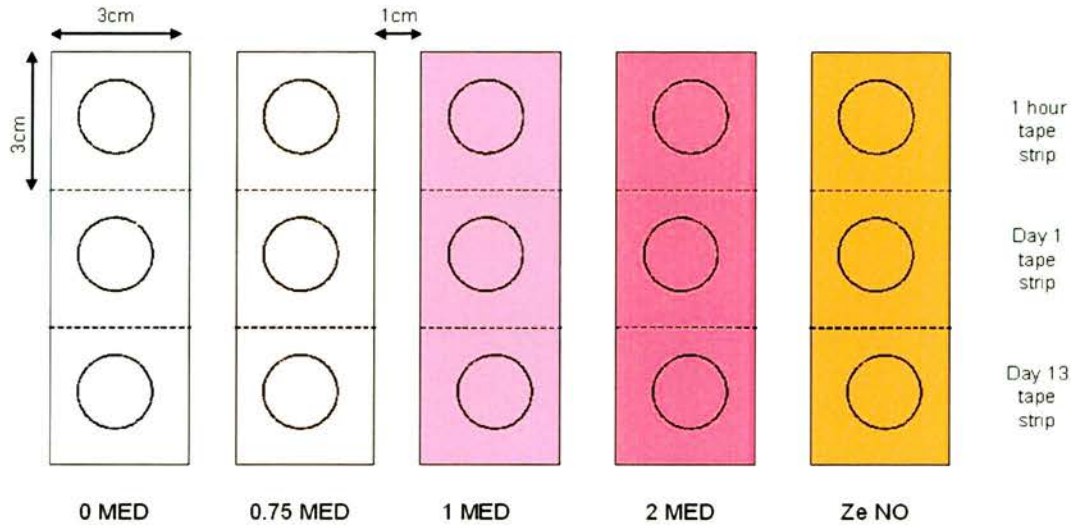


Figure 6.2 *Diagrammatic representation of treatment and tape strip sites of lower back for each subject*

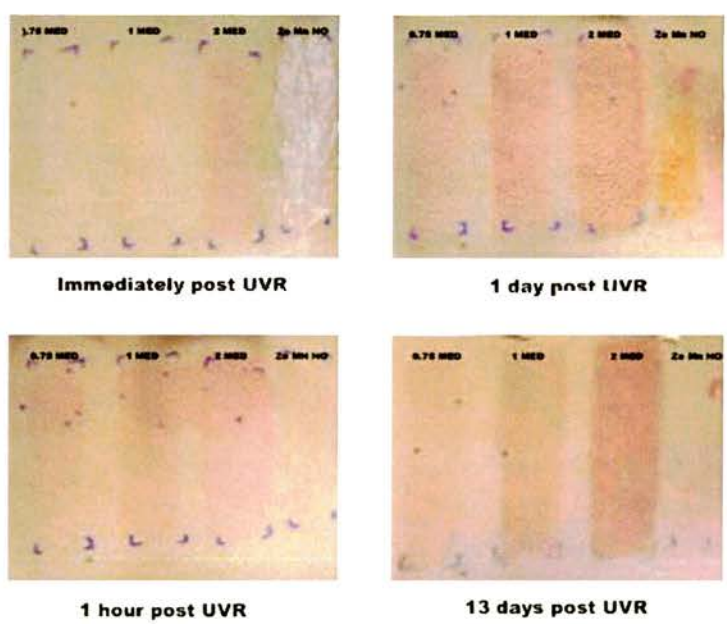


Figure 6.3 *Images of lower back of subject 7 taken prior to tape stripping*

6.3.5. Tape stripping

At 1 hour, 1 day and 13 days post treatment, 20 tape strips were taken from each site (indicated by **O** figure 6.2). Tape stripping was performed as described in Ch2.3.1, figure 2.2.

6.3.6. Amino acid analysis

The concentration of free amino acids (L-ornithine, L-citrulline and L- arginine) were analyzed on tape strip layer 10 using HPLC. All amino acid concentrations were normalised to the protein concentration of the strip. All tape strip analysis was kindly performed by colleagues at Estée Lauder Companies, Oevel, Belgium.

Figure 6.4 *UVB irradiation of lower back during arginase tape strip experiment*

6.4. Results

6.4.1. L-arginine levels in the epidermis do not change in response to UVR or exogenous NO

No significant changes were observed in L-arginine levels in the epidermis, as measured by tape striping, following exposure to UVR. A moderate increase in L-arginine with increasing doses of UVR was seen at two weeks following UV exposure, however this trend was not significant (figure 6.5).

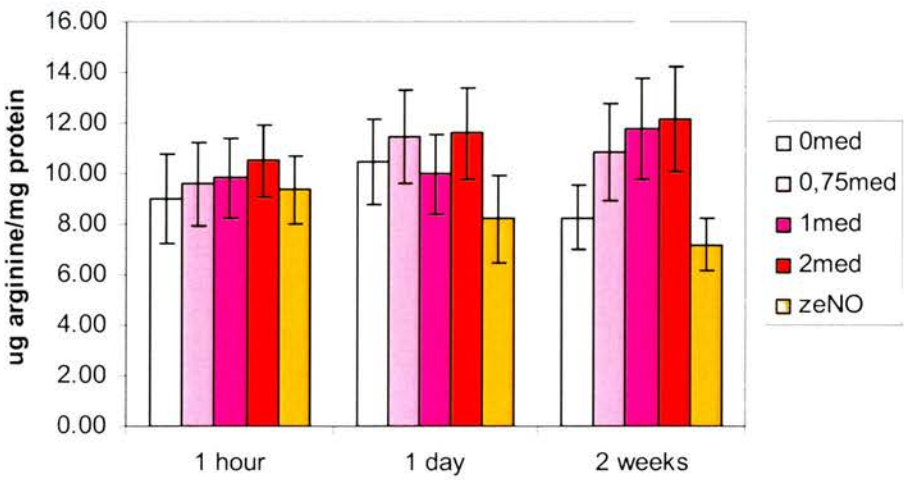


Figure 6.5 *L-arginine concentration as measured from 10th tape strip post UVR or exogenous NO application (n=8)*

6.4.2. L-citrulline levels in the epidermis decrease two weeks after exposure to 2MED UVR

Similar to L-arginine, no significant change was seen in L-citrulline levels in the epidermis following UVR at doses of 0.75 and 1MED, or following exogenous NO donor application. A significant decrease in L-citrulline was observed between 1hour/1 day and 2 week levels following 2MED UVR (figure 6.6).

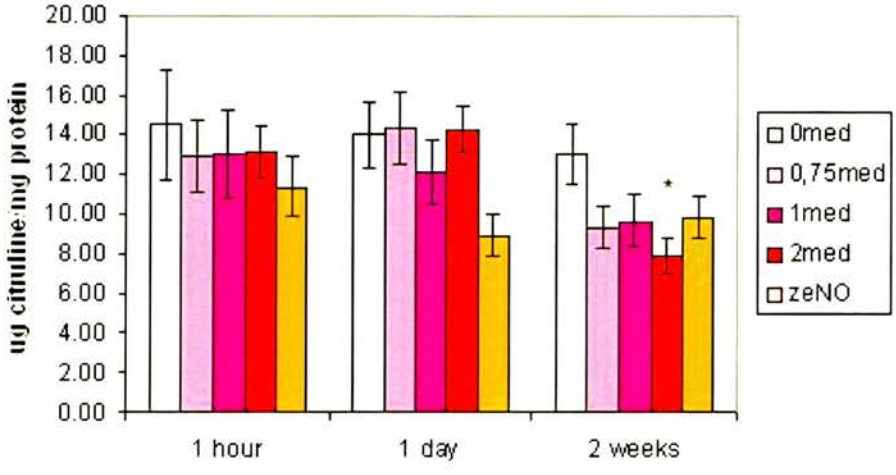


Figure 6.6 *L-citrulline concentration as measured from 10th tape strip post UVR or exogenous NO application (n=8)*

$p<0.05$ 2MED at 1 hour/1 day compared with 2 weeks

6.4.3. A significant dose dependent increase in L-ornithine levels is seen in the epidermis in response to UVR and exogenous NO

Significant dose dependent increases in L-ornithine levels in the epidermis were observed between samples taken at one hour or one day post UVR and those taken two weeks post UVR (figure 6.7). A similar significant increase was seen in L-ornithine two weeks following the application of Ze-NO. Such an increase in L-ornithine is assumed to be secondary to up regulation of arginase, which hydrolyses L-arginine to L-ornithine as part of the urea cycle (figure 6.8).

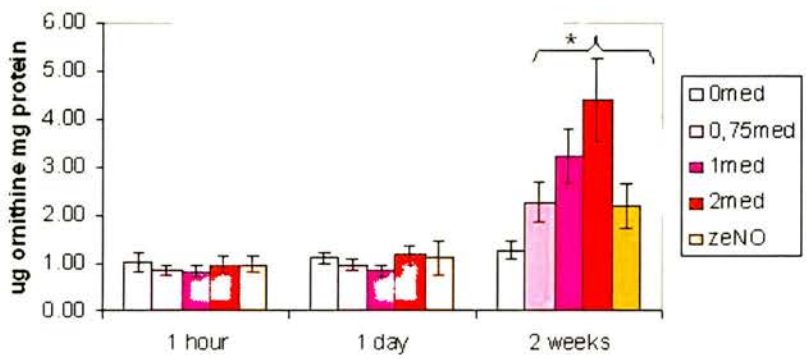


Figure 6.7 *L-ornithine concentration as measured from 10th tape strip post UVR or exogenous NO application (n=8)*

$p<0.05$ 2 week values compared with 1 hour/ 1 day

6.5. Discussion

As has been shown in the preceding chapters, NO is a key player in the cutaneous response to UVR. L-arginine is a common substrate for iNOS and arginase, resulting in NO + L-citrulline or urea + L-ornithine respectively. This experiment provides evidence of a dose dependent increase in L-ornithine in the stratum corneum of human skin 13 days following exposure to UVR. In addition, application of a topical NO donor revealed a similar increase in epidermal L-ornithine, which was comparable to that induced by 0.75MED UVB. These findings provide evidence that

the increase in NO and iNOS following UVR is mirrored by an upregulation in arginase activity, we postulate that this offers an important regulatory feedback mechanism between iNOS and arginase.

Ze-NO was used as a topical NO donor, it is known that this NO donor releases relatively low concentrations of NO(Wheatley et al., 2006). Twenty four hours after topical application of 33% Ze-NO to sun protected human skin, erythema is induced which is equivalent to that seen 24 hours following 0.75MED UVB (figure 6.3).

Interestingly, the increase in arginase activity, as measured by L-ornithine concentration, was not accompanied by a corresponding increase in the other main amino acids in the urea cycle in particular L-citrulline. L-citrulline is the subsequent amino acid in the urea cycle after L-ornithine and also a by product of the generation of NO by iNOS from L-arginine (figure 6.8). I present a number of hypotheses to explain how arginase plays an important role in the L-arginine-NOS feedback loop without concomitant fluctuations in L-citrulline.

The process of tape stripping enables sampling of the superficial epidermal layer, the stratum corneum (figure 2.3a). UVB radiation penetrates to, and exerts its effects on, all layers of the epidermis. The mean epidermal transit time for a keratinocyte to pass from the basal layer of the epidermis to the stratum corneum is 25 days(Weinstein et al., 1984). In taking tape strip samples 13 days following UVR we assume that we will be sampling some keratinocytes that were in the basal layer at the time of irradiation with the majority having been in the lower $1/3^{rd}$ of the epidermis, the suprabasal squamous/spinous cell layers (figure 2.3a). One simple explanation for the findings in this experiment are that the cells which have been sampled by tape stripping were arrested at a specific point in the urea cycle after L-ornithine formation but before its conversion to L-citrulline.

The constitutive NOS isoenzymes eNOS and nNOS maintain homeostatic levels of NO, however high output NO formation as occurs following UVR requires iNOS upregulation. Effective NO generation from iNOS is absolutely dependent upon the delivery of its substrate L-arginine into the cell by CATs. The CAT family is composed of four known isoforms, CAT 1, CAT2A, CAT2B, and CAT-3. CAT-1 is expressed ubiquitously and is present in all skin cells, CAT-2 is expressed strongly in the liver and has been isolated in primary human keratinocytes and dermal endothelial cells, where its expression is up regulated within 24 hours of a Th1 cytokine challenge(Schnorr et al., 2003). CAT-3 is expressed specifically in the brain(Mori and Gotoh, 2000). The cationic amino acids transported by this system include L-arginine, L-lysine and L-ornithine, they share the same substrate specificity, therefore CAT competition (via lysine/ornithine) strongly inhibits iNOS and arginase activity(Suschek et al., 2003a) (figure 6.8). UVR induces an increase in TNFα, a Th1 cytokine, this increase is maximal 24 hours post UVR and may be of importance in inducing CAT-2 expression and allowing adequate delivery of the substrate L-arginine into the cell. Although the majority of CAT-2 expressed is probably transporting L-arginine into the cell some of the increase in L- ornithine induced by UVR maybe secondary to this increase in CAT-2 expression.

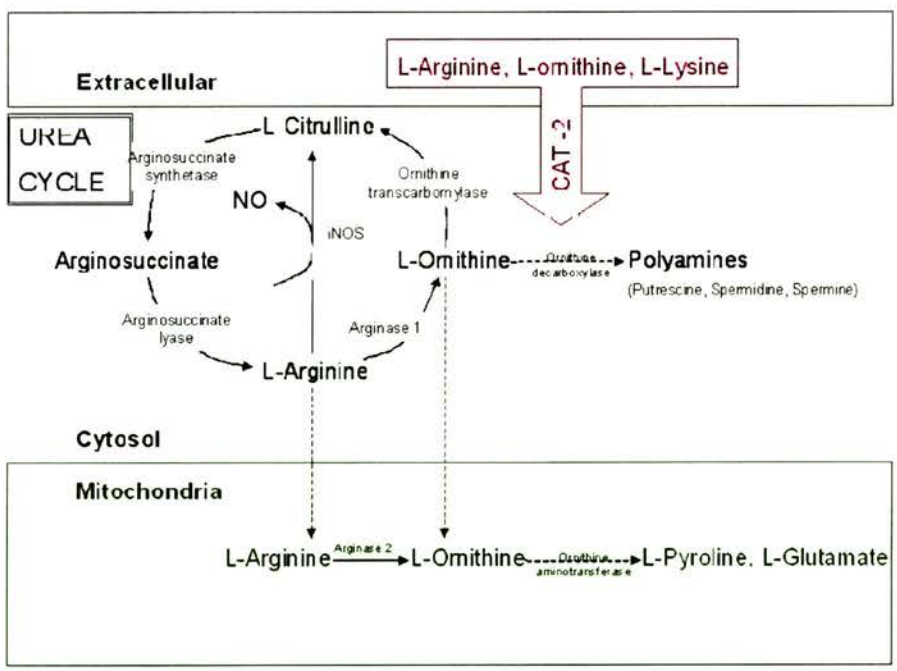


Figure 6.8 ***The Urea Cycle (copy of figure 1.3 page 7)***

Arginases compete with iNOS for the common substrate L-arginine, this enzyme exists as two isoforms: arginase-1, a cytosolic inducible type, and arginase-2, a mitochondrial enzyme. Bruch-Gerharz *et al.,* found that in normal human skin arginase 1 is weakly expressed throughout the epidermis(Bruch-Gerharz et al., 2003). In contrast, Wohlrab *et al.* found arginase concentration to be higher in basal keratinocytes than in the stratum corneum, they found no arginase in the spinous layer (Wohlrab et al., 2002). Li *et al.,* have shown that in bovine endothelial cells production of L-ornithine increased in direct proportion to cellular arginase activity(Li et al., 2001). The data presented in this chapter suggests that considerable arginase activity is present in the epidermal layers of human skin, the activity of epidermal arginase is up regulated by UVR, manifesting as an increase in L-ornithine.

Intracellular compartmentalisation of arginases has an important implication for arginine metabolism in mammalian cells, and may also help to explain the observed absence of an increase in L-citrulline despite an increase in L-ornithine. Such compartmentalisation of arginases has been demonstrated by Li *et al.* who have described the co-localisation of arginase 1 with ornithine decarboxylase in the cytosol, in addition to the co-localisation of arginase 2 and ornithine aminotransferase in mitochondria(Li et al., 2001). Such intracellular compartmentalisation of arginases may itself prevent further conversion of L-ornithine down subsequent pathways such that a rapid increase in L-ornithine is seen as a consequence of high production states, as seen post UVR.

An additional cycle exists between L-citrulline and NO, the 'citrulline-NO cycle', properties of which may also help to explain the apparent low concentrations of L-citrulline despite an increase in L-ornithine. It has been shown in various tissues and cells, including *in vivo* cultured rat macrophages, that arginosuccinate lyase and arginosuccinate synthetase are co-induced with iNOS following cytokine induction(Nagasaki et al., 1996). The co-induction of arginosuccinate lyase and arginosuccinate synthetase with iNOS would ensure that the UVR induced up

regulation of iNOS, with subsequent NO release and L-citrulline generation, does not result in a build up of L-citrulline (figure 6.8).

Despite the complexities of the potential interactions between the different substrates and enzymes of the urea cycle, the experiment described in this chapter shows an impressive dose-dependent increase in L-ornithine/arginase activity following UVR, which is mirrored by exogenous NO. Evidence exists to suggest that in rat endothelial cells the enzyme kinetics favour arginase when up regulated relative to NOS(Berkowitz et al., 2003).

I hypothesise that following exposure to UVA rapid enzyme-independent release of NO takes place from human skin within 20 minutes of exposure, thereafter upregulation of iNOS and CAT-2 (both maximal 24 hours post UVR) results in enzymatic NO release. Consequently UVR induced cutaneous erythema develops, and the increase in NO has an anti-apoptotic role on human keratinocytes(Suschek et al., 1999;Weller et al., 2003) (Ch5.4.1). Subsequent to NOS upregulation arginase is upregulated, this acts as a competitor for the common substrate L-arginine, thus limiting NOS activity. This inbuilt control mechanism in human skin ensures that levels of iNOS in normal human skin return to baseline by 72 hours post UVR exposure(Kuhn et al., 1998). The data presented in this chapter provides new evidence implicating the importance of the urea cycle in control of iNOS and NO generation, it also provides additional evidence to underline the fundamental role of NO in the mechanisms of cutaneous photobiology.

CHAPTER 7
GENERAL DISCUSSION

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7.1. Summary and Conclusions

Knowledge of the physiological importance of inorganic nitrogen oxides is evident dating back to 800AD(Binkerd and Kolari, 1975). However, a massive growth in the interest of the biological effects of NO was not observed until the 1980s. This interest stemmed from the finding that NO release from smooth muscle vascular endothelium has vasodilatory properties(Furchgott and Zawadzki, 1980a;Furchgott, 1986;Ignarro et al., 1987b). Enzyme-dependent NO generation from NOS was described by Moncada *et al.* in 1993(Moncada and Higgs, 1993). Subsequently, three different NOS isoenzymes were isolated, two consitutively expressed isoforms (e/nNOS) and one inducible isoform (iNOS). Constitutive NOS isoforms are regarded as providing regulatory and homeostatic functions, whereas iNOS plays a more pathological role.

With the relative ease of performing IHC staining in skin biopsies, it did not take long before all three NOS isoforms were identified in human skin(Baudouin and Tachon, 1996;Becherel et al., 1994;Bull et al., 1996;Romero-Graillet et al., 1996;Sakai et al., 1996;Wang et al., 1996). A number of groups went on to demonstrate the presence of iNOS in inflammatory skin diseases: psoriasis(Kolb-Bachofen et al., 1994;Sakai et al., 1996), atopic dermatitis(Clancy et al., 1998;Rowe et al., 1997) and LE(Clancy et al., 1998). Since the 1980s NO has become known as a 'double edged sword', the biological roles of this free radical are complex and often vary according to the concentration of NO, the target cell and the microenvironment. This feature is demonstrated by the pleiotropic role that NO can play in both inflammation and apoptosis. This is demonstrated well in psoriasis where iNOS and subsequently NO are elevated in psoriatic plaques, however arginase is also upregulated. Arginase competes with NOS for L-arginine which

results in a relatively low elevation in NO that is sufficient to induce keratinocyte proliferation but not differentiation(Bruch-Gerharz et al., 2003).

NO has been implicated in the cutaneous response to UVR. Inducible NOS is upregulated following UVB exposure, expression can be demonstrated 8-10 hours after exposure and is maximal by 24 hours(Kuhn et al., 1998). Maximal iNOS activity corresponds with UVR-induced erythema, this erythema is abrogated by antagonism of iNOS(Goldsmith et al., 1996). Evidence also suggests that NO plays an anti-apoptotic role in human skin following UVR(Suschek et al., 1999;Suschek et al., 2003b;Weller et al., 2003).

The biphasic nature of NO highlights the importance of *in vivo* translational clinical research in human subjects, in order to accurately investigate the role of NO in the biological situation. Academic dermatologists are well placed to perform such research as the organ of interest 'human skin' is relatively accessible. Initial work began by manipulation of the NO environment with the use of NOS antagonists, unfortunately no NOS antagonist exists that specifically inhibits individual NOS isoforms, and is suitable for use in human subjects. This area of research was made more complex in 2002 following the publication by Feelisch *et al.*, of a method for quantifiying NO-related products(NO_3^-, NO_2^-, RSNOs) in biological samples(Feelisch et al., 2002). It had been known for some time that NO_3^- & NO_2^- are present in the sweat on the surface of human skin(Weller et al., 1996). With the advent of the gas-phase chemiluminesence based method described by Feelisch *et al.*, NO-related products were also quantified in full thickness *ex-vivo* human skin samples(Paunel et al., 2005). Paunel *et al.,* also demonstrated enzyme-independent release of NO from stores of NO-related products in human skin following UVR, this release is maximal within 20-30 minutes of exposure(Paunel et al., 2005).

With the knowledge that NO can be produced in human skin following UVR by both enzyme-dependent and enzyme-independent mechanisms, it became apparent that

manipulation of the NO microenvironment using a NOS antagonist would not be particularly useful, as this method would not fully abrogate NO production. The first experimental chapter in this thesis (Ch3) describes experiments which were designed to investigate the properties of two NO donors, and in particular to demonstrate the nature of Ze-NO. I have shown that Ze-NO delivers physiologically relevant concentrations of NO to human skin without inducing significant inflammation. This is an important observation as previous work by Ormerod *et al.,* demonstrated that acidified NO_2^- when used as a topical NO donor induced significant inflammation(Ormerod et al., 1999a). I suggest that NO is not as potent an inflammatory mediator as was previously indicated, I also propose a number of alternative mechanisms to explain the inflammation seen with acidified NO_2^-.

With the increasing awareness that NO-related products as storage compounds of NO are important in NO biology, and NO-related products in human skin play a role in UVR-induced processes, I wished to further investigate these areas. Using different methods of sampling from human skin: sweat collection, epidermal suction blisters, cutaneous microdialysis and full thickness skin biopsies, I have demonstrated the presence of NO-related products in human skin. Nitrate and NO_2^- are the most common NO-related products found in human skin, sweat and saliva. Consistent with the findings of Paunel *et al.,* I demonstrate that UVA exposure induces release of NO within 30 minutes of the onset of irradiation, as measured by *in vivo* cutaneous microdialysis of the superficial vascular dermis(Paunel et al., 2005). This UVA-induced NO release is reduced by the use of noradrenaline as a dialysate, suggesting that the superficial dermal vasculature is important in the delivery of NO and NO-related products to the skin following UVA exposure. Lundberg *et al*, have shown that the concentration of NO-related products in plasma is determined by dietary NO_3^- ingestion(Lundberg and Govoni, 2004). Data presented in chapter four suggests a relationship between the concentration of NO-related products in plasma and in the sweat and dermis, such that the concentration of NO-related products in human plasma determines one third of the concentration of NO-related products in sweat and two thirds of the concentration of NO-related products in the superficial dermis.

With the knowledge that dietary NO_3^- influences the concentration of NO-related products in plasma, and the evidence that this in turn may influence the concentration of NO-related products in human skin, I postulate that dietary NO_3^- may influence an individuals cutaneous response to UVR.

In vivo murine and *in vitro* human data suggests that NO has an anti-apoptotic role in human skin following UVR exposure(Suschek et al., 1999;Suschek et al., 2003b;Weller et al., 2003). The importance of *in vivo* clinical research in the context of NO has been eluded to constantly throughout this thesis; this is of particular relevance when considering the role of NO in apoptosis. Many authors describe both anti- and pro-apoptotic roles for NO depending on its concentration, the target cell and the microenvironment (Ch1.4). With the use of an inert topical NO donor (Ze-NO), which delivers physiologically relevant doses of exogenous NO, I have demonstrated that NO causes a moderate reduction in apoptosis 24 hours after 2MED UVB. A reduction in stable p53 was also observed in the presence of exogenous NO 24 hours after 2MED UVB. These findings suggest that NO is acting anti-apoptotically in human skin post UVB exposure, I would suggest that a reduction in the accumulation of stable p53 in the presence of exogenous NO is playing a role in this process.

If NO is acting anti-apoptotically in human skin following UVR, is it leading to:

- A reduction in the number of epidermal keratinocytes suffering from significant DNA damage?
- An increase in DNA repair within epidermal keratinocytes?

therefore reducing p53 accumulation and apoptosis. Or alternatively, is NO having more deleterious consequences by inhibiting the important regulatory process of p53 up-regulation and apoptosis, thus allowing the persistence of DNA damaged cells with an increased risk of mutagenesis? In order to answer these questions I examined the role of exogenous NO in UVR-induced DNA damage and repair within epidermal keratinocytes. NO did not influence the accumulation of maximal DNA

damage in epidermal keratinocytes following UVB. Importantly, exogenous NO alone (unirradiated) induced no DNA damage in epidermal keratinocytes. An increase in inter-individual variation in the ability to repair DNA damage in epidermal keratinocytes was seen 30 hours following UVB exposure, in the presence of exogenous NO. This finding may indicate that NO can influence the DNA repair kinetics of epidermal keratinocytes. This remains a complex topic as inter-individual variability in DNA repair kinetics is a widely recognised phenomenon(Bruze et al., 1989;Bykov et al., 1999;Sheehan et al., 2002;Suschek et al., 1999;Suschek et al., 2003b;Young et al., 1996), there remains debate as to which factors are most important in influencing this variability.

Finally, as the evidence for NO as an important mediator in human skin increases, it seems likely that the body has adapted its own control mechanisms to ensure appropriate concentrations of this free radical are available. For example iNOS upregulation following UVB exposure is maximal 24 hours after exposure, but has returned to baseline by 72 hours. L-arginine is a common substrate for both iNOS and arginase, resulting in the production of NO + L-citrulline and urea + L-ornithine respectively. I present strong data which shows that there is a significant dose dependent increase in L-ornithine levels in the epidermis in response to UVR and exogenous NO. This data suggests that following UVR-exposure the upregulation of arginase acts as a negative feedback mechanism and thus contols NO production by iNOS. This provides new evidence implicating the importance of the urea cycle in control of iNOS and NO generation

7.2. Future directions and further studies

The findings presented in this thesis demonstrate many avenues which could be potentially very interesting and useful to follow, in order to further elucidate the role of NO in cutaneous physiology.

Ze-NO is a very effective NO donor, one of its most important features is that it delivers physiologically relevant doses of exogenous NO, and is non-irritating. The use of Ze-NO as an NO donor was vital to many of the experiments presented in this thesis. Although relatively easy to administer, some improvements could be made to this NO donor, which would enhance its potential use as a topical NO donor in future experiments. At present Ze-NO powder is mixed with aqueous cream immediately prior to application on the skin, thereafter the majority of NO is released within the first hour after application. An ideal topical preparation would involve simply the application of one product, with subsequent release being more gradual and sustained over a relatively long period of time (eg hours).

Cutaneous NO-related products are present in human skin. Evidence would suggest that they can be influenced by dietary NO_3^- intake, and that they are strongly involved in the cutaneous response to UVR. It would be interesting to take these suggestions a step further, to determine whether controlling for/adjusting dietary NO_3^- intake directly influences the concentration of cutaneous NO-related products. This could be performed real time *in vivo* by measuring cutaneous NO-related products in the superficial vascular dermis, using cutaneous microdialysis, following ingestion of a dietary NO_3^- load. Alternatively, epidermal suction blisters or full thickness skin biopsies could be used to quantify cutaneous NO-related products , after either controlling for or loading with dietary NO_3^-. Other variables to consider when studying human skin are both an individuals skin type and their tendancy to develop erythema after UV exposure. It would be of interest to study variations in the resting concentrations of cutaneous NO-related products in individuals of different skin type and erythemal sensitivities, and thereafter to identify whether these factors

influence the release of NO from cutaneous NO-related products following UVR exposure. Although much remains to be investigated regarding the role of NO-related products in normal human skin, their role in skin disease is also an important consideration. The ability to quantify NO-related products will be very useful in further assessing the role of NO and NO-related products in skin diseases in which NO has already been implicated, for example psoriasis, atopic eczema and cutaneous lupus erythematosus.

The role of NO in epidermal keratinocyte apoptoisis and DNA damage/repair following UVR is always going to be difficult to study. In addition to the biological variation that each individual brings with them to a study, many additional experimental variables exist. These factors, coupled with the pleiotropic nature of NO, make it vital that further research in this field is performed whenever possible in the *in vivo* situation, using physiologically relevant parameters. As has been discussed earlier, further experiments which control for an individuals dietary NO_3^-, and therefore their ability to produce NO by enzyme-independent mechanisms on UV exposure, may reduce the variability between subjects. Experiments to further elucidate the roles of UVA, UVB and possibly more relevant solar simulated radiation, would also further improve our knowledge of this field. Attempts were made to control for the known biological variation in DNA repair by selecting individuals with the same MED. These individuals exhibited a similar erythemal sensitivity on UV exposure, and the dose of UVR to which each individual was exposed was the same. Improvements could be made by using tighter MED increments, this would provide tighter determination of an individuals MED, such that even closer matching of erythemal sensitivity could be achieved.

With all the experiments which utilized a topical NO donor, 33% Ze-NO was the donor of choice. The concentration selected is the maximum concentration at which Ze-NO powder can be mixed adequately with aqueous cream, and thereafter applied to the skin in a uniform manner. An important consideration in selecting this NO donor lies in its inert properties, in addition it provides physiologically relevant doses

of NO. A number of potentially useful topical donors exist such as S-nitrosothiol-based topical donors, however in my experience these tend to deliver massive concentrations of NO which are several orders of magnitude higher than those seen in the normal physiological situation. In future experiments it would be interesting to determine whether exogenous NO has different dose-effects in the context of inflammation, apoptosis and DNA damage, in the presence and absence of UVR.

Finally, having discussed potential avenues for the investigation of NO and its relationship with inflammation and UVR in normal skin, I would like to progress to looking at skin disease, in particular cLE. Photosensitivity is now a well-established factor in the pathogenesis of cLE, in clinical and experimental settings, and it is included as a discriminating factor in the revised criteria of the American Rheumatism Association for the classification of SLE(Tan et al., 1982). Photosensitivity occurs in 10-92% of patients with SLE, in 50-100% of those with subacute cutaneous LE and in 39-89% of discoid LE(Sanders et al., 2003). In contrast to normal skin, UVR-induced peak erythema and iNOS expression are delayed to between three and 24 days in cLE(Kuhn et al., 1998). Excess apoptosis is also thought to be a central event in the pathophysiology of LE, this may be due to either reduced clearance or increased production(Pablos et al., 1999). Histologically, the characteristic basal keratinocyte apoptosis seen in cLE produces an appearance of 'dermo-epidermal effacement' and an inflammatory infiltrate. Apoptosis exposes epitopes on the nuclear membrane, which are presented by dendritic cells to the immune system, and in the context of the relevant HLA types, leads to the production of autoimmune nuclear antibodies(Caricchio et al., 2003;Gabler et al., 2003). In summary cLE has been shown to be associated with aberrant iNOS upregulation following UVR, in addition the hallmarks of this disease are those which I have studied with regards to NO:

- Photosensitivity
- Epidermal keratinocyte apoptosis
- Inflammation

From an experimental point of view, the disease of cLE can be induced using artificial UV sources, this makes it an ideal disease in which to further study the role of NO in human skin.

7.3. Clinical implications

One of the most obvious clinical applications to stem from this thesis would be the development of Ze-NO as an NO donor for use in the clinical setting. Topical NO donors have already been shown to be mediators in the treatment of cutaneous fungal and viral disease(Ormerod et al., 1999b;Weller et al., 1998), Raynaud's phenomenon(Tucker et al., 1999), diabetic wound healing(Witte et al., 2002a), melanogenesis(Romero-Graillet et al., 1996;Romero-Graillet et al., 1997) and the control of keratinocyte apoptosis following UVR(Suschek et al., 1999;Suschek et al., 2003b;Weller et al., 2003). Further work needs to done, firstly in developing a form of Ze-NO which is easy to apply and releases sustained amounts of NO. Secondly, further investigation of the effects that Ze-NO has on the cutaneous response to UVR, including dose-response studies, are necessary. If exogenous NO at physiological concentrations is found to be reducing UVR-induced DNA damage, and consequently keratinocyte apoptosis, one could envisage its future use in topical sunscreen preparations.

An additional implication of my hypotheseses regarding the protective role of both enzyme-independent and enzyme-dependent NO in human skin has implications for health promotion. Should it be found that dietary NO_3^- does directly influence cutaneous stores of NO-related products, a logical progression would be to advise a NO_3^- rich diet to individuals who seek to expose their skin to UVR.

7.4. Summary

In summary, I have studied the role of 'NO as a regulator of apoptosis and inflammation in human skin following UVR'. I present Ze-NO as a clinically and experimentally useful NO donor which has essential non-irritating properties. I have shown that human skin, the largest organ in the human body, acts as a store for NO-related products both in sweat on the skin surface and in the skin itself. Evidence suggests that these stores correlate with the concentration of NO-related products in plasma, which are in turn determined by dietary NO_3^- intake. The demonstration of rapid enzyme-independent release of NO from stores of cutaneous NO-related products within 20-30minutes of UV exposure, is likely to be important in the cutaneous response to UVR. This rapid release of NO bridges the time gap before the upregulation of iNOS and enzyme-dependent NO release takes place. It seems that NO release from human skin following UV exposure has an anti-apoptotic role, however further work needs to be done to elucidate its effects on epidermal keratinocyte DNA damage/repair. Finally, it is likely that the human body has an inbuilt regulatory mechanism to ensure control of NO production following UVR. Evidence suggests that a competitive feedback loop exists between arginase and iNOS for the common substrate L-arginine, this provides control over NO production and ensures that iNOS returns to baseline levels within 72 hours of UVR. These findings add greatly to the knowledge of NO and its role in human skin, in addition they highlight many exciting potential avenues for future research in this field.

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8. References

8.1. References

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